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Will the DNA guidelines wither away?

On both sides of the Atlantic, the regulations for the control of recombinant DNA research are being substantially relaxed (see *Nature* 27 September and 2 October). At the present pace of about one major relaxation every two years, it will not be long before the regulations have virtually disappeared. Both in Britain and the United States, the regulatory authorities (the National Institutes of Health and the Genetic Manipulation Advisory Group) have decided that a substantial part of their work shall be in effect delegated to local safety committees, working within institutions (in the United States) or laboratories (in the United Kingdom). The categories of hypothetical hazard into which experiments of different kinds have been slotted in the past five years, have also been changed once again, with the result that a large proportion of the experiments still formally under the control of the regulatory bodies will now be exempt. Is it time that governments began to think of what should be done about recombinant DNA research say two years from now, when there is almost nothing left to regulate?

Some recrimination is unavoidable. Many impatient people, who have regarded the past period of regulation as an intolerable and unnecessary impediment to their work, will make fun of the way in which the guidelines were hardly complete before the process of dismantling them began. Some, alas, will take out their anger on hapless Professor Paul Berg, who is rightly or wrongly thought to have been the instigator of the movement to regulate recombinant DNA research — or to have provided governments with an excuse to do so. Then, no doubt, in other DNA laboratories, there will be some who will continue to complain that the genetic manipulators are being given an even wider licence to pervert the course of human evolution and so on. Both strands of complaint will be misjudged. Both camps should be grateful that the regulatory bodies have somehow kept the peace in the past five years.

The truth is that at the outset, five years ago, a great many serious people, many of them molecular biologists, were genuinely alarmed at the risks that recombinant DNA research might bring. The risks were always conjectural, but they could not be confidently equated with zero. But, in the past five years, solid evidence has accumulated that many of the conjectured risks are insubstantial. *Escherichia coli* K12 does not, after all, survive in the human gut, while the frequency of recombination of K12 with wild-type *E. coli* or other organisms has turned out to be too small to sustain the conjecture that altered genes might become permanent components of the gut flora. The recognition, since the guidelines were first drawn up, that introns are more the rule than the exception in eukaryotic genes has decreased the chance that a plasmid carrying a foreign piece of DNA will actually become a source of foreign and possibly damaging protein. Some of the more fanciful conjectures of the past — the possibility that engineered bacteria or viruses might disturb the immune system, for example — have not been much affected by discoveries in the past few years, but they were always conjectural conjectures, so to speak. In general, the discoveries in the past five years which bear on the safety of genetic manipulation support the argument that genetic manipulation is unlikely to create threats to people, other animals and plants that would not have arisen in the course of evolution. This does not imply that all risks can now be disregarded, but merely that most of the risks originally conjectured are less immediate than was thought.

The systems set up by most governments for regulating recombinant DNA research have served a useful purpose in the

past five years. They have provided the general public with a sense that everything possible has been done to avoid or to diminish risk. Most of the committees responsible for administering the guidelines have functioned well. Some of them (the NIH committee, for example) have even functioned publicly. From the public point of view, they have also worked cautiously. Restrictions have been removed, and types of experiments recategorized, only after the wisdom of doing so had been fully substantiated. (The recategorization by NIH of certain animal virus experiments in the summer of 1978 may have been an exception.) One result may be that people have not been able to get ahead with research as quickly as they might. Another has been that there has never been a legitimate cause of complaint by the general public against the practice of genetic manipulation. Such warnings of impending catastrophe as there have been have cut very little ice.

The benefits of this climate of civility in the past five years far outweigh the inconvenience and the delay which some have suffered. The cost of the special laboratories which have been built to contain experiments no longer required to be carried out in such stringent circumstances is a small price to pay for the relative peace and quiet there has been. (The institutions saddled with them may take some comfort from knowing that if they become white elephants, they may also be taken as marks of their prestige in the late 1970s.) There is yet a chance that the case of recombinant DNA research may become a useful model of how new technologies might be introduced.

So what lessons can be learned from the experience of the past few years? And what does the future hold for recombinant DNA research?

The first thing to appreciate is that the time has not yet come to make a bonfire of the regulations. The main result of the most recent relaxation is, in effect, to license laboratories and their safety committees to carry out experiments in the lowest categories of supposed hazard. Categories, and prescribed containment, will remain for others, and it will be some time before further relaxation is possible. Looking ahead, some kinds of recombinant DNA experiments that people wish to carry out would in future (as now) fall within the scope of the guidelines which regulate experiments with dangerous pathogens even if the recombinant DNA guidelines were abolished. Problems of scaling-up from the laboratory to the manufacturing scale also remain, and the regulatory committees are rightly preoccupied with them. (One useful beginning would be to find a better distinction between the first and the second than the entirely arbitrary supposition that 10 litres of any culture constitutes "scaling-up".) Ultimately, however, procedures on an industrial scale will be regulated by the statutes which apply to occupational safety, so that that the withering away of the guidelines for recombinant DNA will not provide manufacturers with an entirely free hand.

Thus there is a prospect that the DNA guidelines may, at some time in the future, be subsumed in other and long-standing sets of guidelines. What about the experiments in the P2 and P3 categories (NIH terminology) that will continue to be regulated by DNA guidelines at least for the next few years. Even here, the regulatory authorities should move as quickly as they can towards some version of a licensing system — one in which laboratories (and their safety committees) take the first responsibility for deciding what degree of containment is appropriate for which experiment, and then provide the regulatory authorities with a



report on what they have been doing at least once a year. Apart from the benefits that would follow for DNA researchers, questions of industrial secrecy would also by such a step be simplified enormously. In moving towards such a system, however, the committees should be much more concerned than in the past to coordinate their practice internationally. The unfortunate case of Dr Samuel Kennedy of the University of California (see *Nature* 18 September) may be a vivid illustration of the trouble caused when governments follow different standards. Nobody can know at this stage what part international competition played in the affair, but the German guidelines were at the time less stringent than the American — and Dr Kennedy's chief rivals in the study of the arbovirus genome are in West Germany. Is it not high time that the regulatory committees recognized that such ironies will persist as long as they decline (as they have almost studiously done) to talk to each other directly?

The question remains of how best to deal with problems like those occasioned by genetic manipulation which are certain to arise again. In retrospect, in countries as different as Britain and the United States, the committees set up to hold the ring between the public and the molecular biologists have served reasonably well. If anything, the molecular biologists have been more frustrated than the public, which is only right and proper. On balance, especially in the early days, it would have been helpful if the proceedings of the British group responsible for the guidelines had been carried on in public as, for most of its time, the corresponding American committee has been required to do by the Freedom of Information Act. Many incipient misunder-

standings about the functioning of the Genetic Manipulation Advisory Group have been consequences of the secrecy imposed on it under the Official Secrets Act as well as by the conventions of Whitehall. People may rightly ask what kind of public assurance can stem from the proceedings of a secret committee. Even so, the committee has functioned better than many expected.

The most obvious deficiency of the past few years is that, faced with a problem of assessing the safety of a new and unexplored laboratory technique, the responsible committees (and the scientific community itself) did very little to provide the data on which objective assessments of safety might be based. It is true that people have fed each other quantities of *E. coli* from time to time, and there have been a few half-hearted attempts to see whether genes incorporated into bacteria can find their way into the somatic cells of laboratory animals. Few opportunities have been seized for looking into the ways in which bacteria carrying foreign genes might actually damage their adventitious hosts. The customary explanation, during most of the past five years, is that the people most competent to carry out such experiments have been too preoccupied with exploiting the new techniques whose safety was called in question. With a few conspicuous (and honourable) exceptions, those with the most vivid interest in demonstrating that their techniques are as safe as they now appear have been content with arm-waving. They, as well as the rest of us, are lucky that things have turned out as they have, and that the general perception of the risks of genetic manipulation has so quickly, and painlessly, moderated. It could have turned out quite differently.

McNamara's fond farewell to his bank

Who would think that the president of a large automobile manufacturer turned US Secretary of Defense at the height of the Vietnam War would turn out to be a superb president of the World Bank? The credentials, impressive enough, are on the face of things unfitting. Yet the governors of the bank plainly knew what they were doing when they appointed Mr Robert McNamara to his present post nearly fourteen years ago. During that period he has managed both to dramatize the cause of economic development in the poorest countries of the world and then to make the World Bank itself into a powerful instrument for prosecuting that cause. He has, in other words, justly earned the right to make the fierce complaints which marked his valedictory address to the members of the bank last week. And he is right, of course, to note that the cause of development has gone sour in the past few years, right to complain that many industrialized countries (Britain was singled out) are reducing their contributions to overseas aid, right to complain that the OPEC surplus states should carry a much greater burden and right to fear for those who carry the true burden of development:

"We do not see their faces, we do not know their names, we cannot count their number. But they are there. And their lives have been touched by us, and ours by them."

In retrospect, Mr McNamara's most cheerful memories will no doubt be of the early 1970s, when he was stumping the world urging governments that they should not forget the "poorest of the poor" and at the same time persuading the members of his bank that their funds should be invested as often in social infrastructure as in mammoth capital projects. Even then, however, it was an uphill struggle. With a few rare exceptions (Sweden, for example), most industrialized countries never consistently came near the United Nations target for overseas aid of 0.7 per cent of GNP. Since the increase of the price of oil, performance has worsened — and the World Bank has become even more important as a source of multilateral aid. The explanation is, unfortunately, all too simple. Industrialized governments have become obsessed with domestic problems, many of them caused by the increased price of oil. But at a time when voters are being asked to pay more tax or to do without public services, governments are plainly unwilling to prosecute

the cause of development. The Brandt Commission was upbraiding them for their neglect earlier in the year. Mr McNamara has now added his complaints.

The complaints are fair, but they will not be listened to with much care. One trouble is that governments are preoccupied with other things. A more serious difficulty, which both the Brandt Commission and Mr McNamara overlook, is that the cause of development has gone sour because relations between the industrialized states and the rest of the world have themselves gone sour. Although most oil consumers recognize by now that the crude oil prices of the 1950s and 1960s were unfair to the oil producers, they have not enjoyed the humiliations of the past few years, or the economic upheavals that have gone with them. And they will not forget them. In such circumstances however, it is natural enough, if wrong, that the Western oil consumers should be saying that if any group of states should take responsibility for helping with economic development of the poor countries, the OPEC states are the obvious candidates. So (as Mr McNamara says) they are. Unfortunately the OPEC states are even more preoccupied with their own problems than are the industrialized states of the West. Since there is no prospect that the members of the Warsaw Pact and its adherents will produce the kind of help that the developing countries need, it is no wonder that Mr McNamara's speech was so gloomy.

So is all hope lost? Not quite. Or perhaps not yet. There are a few clouds with silver linings. The OPEC states have somehow to manage their surpluses, usually by asking other people to do that on their behalf. There may be some pickings for development there. Better, even the past two sad decades have shown that some developing countries can actually develop, and make good. The example of India is not a joke. Better still, it appears now to have been understood in the West (but also in the developing world) that development assistance is not the provision of capital funds but help with the process of development, "infrastructure" as the saying goes. Sometimes this means teaching businessmen how to do accounts, politicians how to be ministers and children how to read. Building roads is by these standards capital-intensive. If only Mr McNamara had had another fourteen years, he might have been able to demonstrate what many suspect — that the most imaginative development is cheap.

Melanoma increase in radiation labs?

Livermore data may be significant

Washington

US Department of Energy officials remain baffled about the cause of an abnormally high incidence of malignant melanoma among employees of the University of California's Lawrence Livermore Laboratory, one of the nation's centres for nuclear weapons research.

An advisory board report released in Washington last week confirmed findings suspected since 1976, and substantiated earlier this year by California's Department of Health Services, that the incidence of skin cancer among laboratory employees was three to four times the national average.

The California study showed that between 1972 and 1977, 19 white workers at the laboratory had developed malignant melanoma, compared with between 4.6 and 6.4 cases expected using national statistics, a result which it said was "very unlikely to have occurred by chance".

At the request of the Department of Energy, the data were reviewed by an advisory board chaired by Dr Arthur Upton, then director of the National Cancer Institute. The board found no flaws in the Californian study and revealed that the increased incidence seems to have continued, with eight more cases of melanoma being reported up to June 1980.

However, the advisory board reports that preliminary efforts to explain the excess have not succeeded in implicating any specific cause. It says that the occupational safety, industrial hygiene and medical programmes of the laboratory appear to be well conceived and well conducted, and that additional protective health measures "appear to be unwarranted".

It also lists a range of possible environmental causes, from occupational exposure to chemicals to details of individual lifestyles, which it says should be studied in more detail to get to the root of the problem.

"Part of the difficulty is that there may be no problem at all. We still don't really know if this thing is real; it may be just a statistical fluke" stated one member of the board last week, pointing out that the continued high incidence could be the result of people looking for — and finding — melanomas many years earlier than they would otherwise be detected.

Although a relatively rare form of cancer, the incidence of malignant melanoma has been increasing faster than almost any other cancer in recent years. In

San Francisco, for example, the number of cases rose from 5.8 to 11.2 per 100,000 between 1970 and 1975, and similar figures have been reported for other parts of the country.

One fashionable explanation is that, because the greatest increase has been found among fair-skinned professionals, whose work tends to keep them indoors, it may be caused by intermittent exposure to ultraviolet radiation, for example as a result of excessive sunbathing or other outdoor activity.

Preliminary examination of the occupational histories of those contracting skin cancer has failed to reveal any obvious common pattern of exposure to particular chemicals or forms of radiation, and the board's report points out that although malignant melanomas have been found in an irradiated person, epidemiological studies have failed to produce convincing evidence that tumours may be induced in the skin by ionizing radiation.

However, the possibility that occupational exposure to, for example, internal emitters and ultraviolet radiation may contribute to the aetiology of melanoma is one of several avenues that the laboratory intends to explore further. "It is just possible, because of changes in lifestyle such as improved nutrition, that we have managed to increase our risk of cutaneous melanoma to the point where, although radiation was not a cause in the past, it might have become one today", says Professor John A.H. Lee of the

Department of Epidemiology at the University of Washington in Seattle.

In a memorandum submitting the board's report to Energy Secretary Charles Duncan, the Department of Energy's Assistant Secretary for the Environment, Dr Ruth Clusen, suggests that further studies by both the laboratory and the state Department of Health Services should be supported.

The latter has already proposed a major follow-up programme, which is being reviewed by the department that will take a detailed look at the particular social characteristics of the Lawrence Livermore workforce. A parallel study may also be carried out at another Department of Energy site.

The Lawrence Livermore Laboratory is operated by the University of California under contract from the Department of Energy. Its involvement in weapons research — and the resulting need to keep much of the research programme secret — has been the source of sharp controversy over whether its links with the university should be maintained.

Last year the university's board of regents rejected a call from California Governor Jerry Brown that all weapons-related research be moved to the Los Alamos Laboratory, and last month the regents agreed to start negotiations with the Department of Energy for a new management contract for the laboratory. The current contract expires in 1982.

David Dickson

EEC worries about veal and hormones

The massive veal market in Europe, consuming eight million calves a year, was on the verge of collapse this week following scares in France and Italy over the levels of an artificial growth hormone — diethylstilboestrol — in the meat. Diethylstilboestrol (DES) is a synthetic oestrogen which caused vaginal cancers in the daughters of mothers who were prescribed it (in large doses) as a protection against spontaneous abortion in the 1940s and 1950s. Its use is banned in the United States and in most countries of Europe, but a black market has sprung up because this chemical can add 5–15 kg to the weight of a calf and thus double a farmer's profits.

In Italy, the trouble began on 4 September, when the Ministry of Health placed a temporary ban on twenty-two brands of baby foods containing veal. A fairly crude bioassay had shown samples to contain an active oestrogen, probably DES. On 22 September a local magistrate in Latina, south of Rome, used the wide powers of Italian magistrates to impose a nationwide ban on the sale of veal. The Rome laboratory of the Istituto Superiore

di Sanita, the technical arm of the Ministry of Health, is now making mass spectroscopic measurements of the hormones isolated from the baby foods to check for DES. In the view of a British scientist, the levels are probably "quite high" or they would be undetectable by the original method.

In France, pressure began independently from the left-wing trades union group, Paysans Travailleurs, whose members had accumulated evidence that French veal producers were using the synthetic hormone. Paysans Travailleurs then recruited help from the consumer group, the Union Fédérale des Consommateurs, which called for a boycott of veal in the September issue of its magazine, *Que Choisir*. Subsequently, in a rare enforcement of French law on the matter, a 29-year-old pharmacist and veal producer was jailed.

Pressure from farmers and other interests has forced the Italian magistrate to relax his ban somewhat, but sales of veal have still fallen by 50–60 per cent in both France and Italy, and the export of calves

from Britain — where hormone use is controlled under the 1968 Medicines Act but not banned — has fallen to a trickle in a business normally worth £500,000 a week.

Last Tuesday agriculture ministers of the European Community tried to take a grip in the affair by promising that Commission officials would devise a scheme — within a couple of months — for uniform testing of hormone levels in meat throughout the

EEC hormone law

Belgium, Denmark, France, Italy and the Netherlands already have a total ban on the use of natural or artificial hormones, but avoidance is thought to be commonplace. Belgium, France and West Germany take the view that a partial relaxation of the ban in favour of natural hormones — oestradiol and testosterone — would stop the abuse of the synthetic hormone which can be injected directly into the muscle as an oily, crystalline suspension, and is not easily detected by visual inspection of the meat after the animal is slaughtered; other hormone preparations, however, are administered as pellets implanted prominently in the ear. The ear is discarded at the slaughterhouse, but injected muscle will find its way straight to the table — or the baby food.

West German legislation, at present, distinguishes a list of hormones which may be administered — the positive list — and a list which may not — the negative list. On the latter are those which have an oral oestrogenic activity of more than one-fifth that of DES, so banning DES, the related hexoestrol, and two other artificial oestrogens. On the positive list at less than one-fifth the activity are the natural oestradiol and the artificial agent zeranol.

In Britain, the Medicines Act allows licensing of hormone preparations by the Veterinary Products Committee, under the Ministries of Agriculture and Health. Under these licences zeranol and hexoestrol are on free sale to farmers; and Hoechst's Finaplix (an androgen used in beef production) and Implixa (used for veal) administered by vets. The drugs may not be administered within at least 60 days of slaughter (although the drugs firms are pressing to reduce this period). The Pharmaceutical Society has the statutory authority to police the proper use of the drugs.

DES is no longer used in veal or beef production in Britain, although it is used occasionally in pig farming. One veal producer for the small — 70,000 head a year — British home market, Philip Paxman of Volac Ltd, said last week that he had stopped using hormones at all because of public pressure; and they are rarely used in the 300,000 head export market, he believes, because calves are shipped at 4–6 weeks old for fattening on the continent.

EEC. Despite great pressure from Italy, however, the ministers drew back from imposing a total ban, but will not easily agree on the directive they have asked for.

UK specialists in the use of sex hormones in farming argue that any proposed European legislation should distinguish between artificial agents and natural steroids (like oestradiol and testosterone); and that the use of natural steroids should be limited to tissue levels of around $1 \text{ in } 10^9$ — similar to those found in untreated farm animals. For the synthetic anabolic agents, and their frequently unknown residues, they should be shown to be non-toxic and non-carcinogenic before licensing.

European consumers unions, however, represented in Brussels by the Bureau Européen des Unions Consommateurs (BEUC) are pressing hard for all hormone use for meat production to be banned. It is too difficult for a policing authority to distinguish natural from unnatural hormones, says BEUC, so both should go. If agreed, this would have a big impact on beef production in the UK, which in the past few years has come to depend on hormone supplements for its profits, and would cause pressure to increase the use of antibiotics which have a similar effect on weight gain.

And perhaps Italy should worry more about olive oil than veal. According to one pharmaceutical company spokesman, vegetable oestrogens — of one-hundredth or so the bioeffectiveness of oestradiol, are present in olive oil at such a high level that the consumption of 50–100 g of olive oil a day would reverse an ageing woman's post-menopausal changes. But then if Italy can ban veal, it can probably ban olive oil too.

Robert Walgate

Polish farming

More meat to eat?

Agriculture is an extremely sensitive issue in Poland. After all, it was the announcement of increases in the price of meat products on 1 July that led to the recent wave of industrial unrest. Speaking last week at the Olsztyn Agricultural-Technical Academy, the Polish President, Henryk Jablonski, explained that what Polish agriculture needs is a robust, up to date and consistent farm policy, which will take into account the needs of development, technical equipment and the "fullest possible utilization" of scientific achievements. In particular, there is a pressing need for people of "considerable professional qualifications and social dedication" to implement research and guide agricultural development.

Under clause 6 of the Gdansk accords, the Polish government undertook to create "lasting prospects for developing peasant family farms which are the foundation of Polish agriculture". The greater openness of information stipulated by clause 3 of the same accords means that for the first time

official figures comparing the performance of state and private agriculture are being made available to the general public. Some 75 per cent of Polish agricultural land is still in private hands — some 3 million family farms of which barely 400,000 exceed 10 hectares. A minimum of 15–20 hectares is considered necessary for really efficient farming in Polish conditions, hence the large state farms would be expected to have the most advantages particularly because the state sector receives 64 per cent of all investment in agriculture. Yet, according to the official figures for 1979 (quoted by Leszek Chmielowski in *Zycie Warszawy*, 23 September), the state sector uses 2.5 times more chemical fertilizers and 2.6 times more concentrated feedstuffs to give the same amount of produce.

Chmielowski suggested that subsidies to state farms should be reduced, administrative costs minimized and unrealistic plans phased out. The land fund (largely made up of former private farms handed over to the state in return for a pension by elderly farmers without an heir eligible to succeed them) should not, said Chmielowski, simply be used to increase the area of (often unproductive) state farms, but should be made available to all private farmers who wish to expand.

Nevertheless, the members of the new "self-governing" farmers' union feel that Chmielowski's proposals do not go far enough. He stressed, for example, that the state sector, properly organized, still has "an important and growing role to play". But as Wieslaw Kecik, a young intellectual temporarily coopted as an "assistant" to the new union, explained, the greatest deterrent to the ambitious private farmer is his lack of security of tenure. Official policy, since the early 1970s, has aimed at "collectivisation without loss of production". If the state sector is to grow, the private farmers feel, it can only do so at their expense.

Moreover, said Mr Kecik, there are considerable price differentials in favour of the new sector. The private farmer must pay more for all essentials, and receives lower prices for his produce, except for a few "commercial" crops such as rape-seed and flax. Abolition of such inequalities, and the guarantee of land tenure, "must be", said Mr Kecik, "basic postulates of the new self-governing farmers' union".

The day after Chmielowski's article appeared, the Prime Minister, Josef Pinkowski, addressed a meeting of farmers and agricultural workers in terms of apparent reassurance. After calling on all those engaged in agriculture and its ancillaries, including research, to increase their efforts, Pinkowski indicated that the government would raise funds to create favourable conditions for "the whole of agriculture — all productive private farms". "We should like every farmer to feel that his work is secure", he added. "We want him to know that his intentions and production plans, his desire to

modernize his farm and to increase its productivity, will meet with continuous support from the State."

Last Saturday, at the meeting of the Party Central Committee, First Secretary Stanislaw Kania was even more forthcoming. "Individual peasants carry the Society", he said. "We shall ensure that every peasant is certain of his ownership and his farming tenure."

The next few months should show how far practical policy implements these assurances. **Vera Rich**

Kopek interferon

Last week, the Soviet news agency TASS announced that interferon can be purchased in any Soviet drug store for 56 kopeks per ampoule. The Soviet Union, said TASS, was the first country to produce interferon commercially, and for more than a decade Soviet doctors have been using interferon to prevent influenza and other virus diseases of the respiratory tract. Soviet doctors, said TASS, also use interferon against leukaemia and viral skin diseases. A patent for interferon production has been purchased from the Soviet Union by Japan.

What the TASS announcement does not make clear, however, is the extremely low dosage in the 56 kopek ampoule. Samples analysed in Western laboratories contained dosages of the order of 1,000 units or less. The market price of Finnish interferon is US \$25.00 per million units. At 64.4 kopeks to the dollar, Soviet interferon is therefore by no means competitive.

A careful reading of the TASS interview with Dr Valentin D. Solov'ev, moreover, reveals that studies on leukaemia, skin diseases, and also the veterinary use of bovine and porcine interferon, are still at the experimental stage. It is only the interferon nose-drops, which Dr Solov'ev himself pioneered in 1968, that are in regular use. However, Western experts find it difficult to understand the discrepancy between their results and those of Dr Solov'ev. In the West, interferon has been used successfully to prevent respiratory virus infections, but only at high doses and in patients with little or no natural resistance.

As far as mass prophylaxis is concerned, as the *British Medical Journal* observed recently, since interferon, like any intranasal liquid, has only a short half-life in the nasal cavity, but needs long-term contact with the nasal epithelial cells in order to make them more resistant to virus infection, "it is therefore difficult to understand how... nose drops containing a low titre of human leukocyte interferon could cause the degree of protection under epidemic conditions reported by Dr Solov'ev".

Vera Rich

Gulf pollution

UN's other worry

War is the greatest immediate threat to the Middle East — but the destruction of its fragile water supply, the Gulf itself, must be next. At the end of this month local marine scientists meet in Kuwait, next door to the Iraq/Iran conflict, to pledge research spending of up to \$5 million over two years to find out exactly what is happening to the waters of the Gulf.

The long-term development of the region, the Gulf States have recently recognized, depends on proper environmental management of the Gulf and its coastal regions; yet little is known of its ecology, oceanography or meteorology. The water of the Gulf is needed for agriculture, coastal industries, and for drinking, yet the highest density of tankers in any ocean discharges its ballast into it, and many industries such as desalination plants with their associated chloralkali works (using mercury catalysts), aluminium smelters (emitting hydrogen fluoride), fertilizer plants (emitting ammonia), and refineries (sulphur dioxide and hydrocarbons) belch out completely uncontrolled emissions. Already seafood is frequently tainted with oil, and city environments have

Gulf States. Sewerage networks and treatment plants will serve the whole of Kuwait by 1982, Qatar by 1985. But Bahrain's system is not planned to be complete until 2008, and Saudi Arabia's treatment plant on the Gulf is overloaded.

Hence the effort, catalysed by the Regional Seas Programme of the United Nations Environment Programme (UNEP) to undertake research into the sources and impact of pollutants. At present there are relatively few institutes in the region capable of doing the research, but the picture — UNEP estimates — will be "drastically changed" within a year. UNEP, with its experience in the Mediterranean pollution programme, will help with training, but the Gulf States will jealously guard their technical independence: they have no wish for "expert" teams descending from outside.

At Kuwait, seven research projects will be discussed. The most likely to be funded concerns oil: what are the principal sources of oil pollution in the Gulf, how is the oil distributed, and what are its effects on human health and the marine and coastal ecosystems? But to understand this question, the basic physical and chemical oceanography of the Gulf must be studied, and the biological balance of the sea understood.

Meteorology is also important. The



Kuwait Harbour, twentieth century skyline

deteriorated markedly in the past two or three years.

There are also at least 20 major industrial development regions around the Gulf coast, enjoying the world's greatest rate of development, and populations are flooding to these areas from the interior. Often the legal or institutional mechanisms do not exist to control pollution. And all this is threatening a sea on average only 35 metres deep, which evaporates more water than it receives from river run-off. (The Indian Ocean contributes the balance, through the Straits of Hormuz.)

Sewage is coming under control more rapidly than other pollutants, because of the existence of international contracting companies which are selling systems to

"Shamal" winds blow dust which sediments much of the surface oil; Kuwait suffers frequent calms and temperature inversions which trap airborne emission. Coastal mining is affecting wave erosion and sedimentation patterns, which could in turn affect access by the supertankers; Gulf currents at all depths must be measured. What is the residence time of water in the various regions of the Gulf? This question affects the concentrations that pollutants might be expected to reach.

Other studies would measure the levels of heavy metals in economically important marine organisms; and investigate the distribution and sensitivities of plankton, the lowest level of the food chain.

Robert Walgate

Nuclear equipment

Secrets lifted

Washington

US physicists concerned that their work may become subject to national security restrictions because of its potential military value to foreign powers will soon have a public list to turn to for guidance. The list should also be an invaluable guide to foreign governments wishing to find out what the United States considers sensitive in nuclear technology.

The main purpose of the list will be to provide officials in the Department of Commerce with guidance on what controls should be applied to technology exports in sensitive areas such as nuclear energy. The full list is being drawn up by the Department of Defense, as required by the Export Administration Act of 1979. Last week, as part of this exercise, the Department of Energy (DoE) published in draft form a table of what it calls "energy-related militarily critical technologies".

Details are given of the major components of all technologies related to the exploitation of nuclear energy, from nuclear fuel processing and fusion reactors to security techniques. For each "critical" technology, the department identifies "keynote hardware" necessary for its successful implementation. It describes not only the military and the civilian uses of the technology but also the foreign countries in which there is evidence that the technology is currently available.

The proposed classifications are only preliminary, and are open to public comment so that a more refined list can be produced. The department hopes that the list will be valuable in calling attention to areas of concern, but publication of the list may also prove to be controversial. Some countries, particularly in Europe, have previously argued strongly that as much information as possible about the components of nuclear technology — particularly those relevant to its military

applications — should remain secret. They claim that publication could provide a shopping list for non-nuclear countries interested in obtaining nuclear weapons, making the job of limiting proliferation more difficult.

Department officials said last week that all the information in the list had been taken from the open literature. They claim that, although they have indicated where a country has the capability to develop a technology, this does not imply that it has done so, since much of the information was inferred from data reported in the scientific and technical literature.

The table accompanies a broader but less detailed list of militarily-sensitive technologies published in the Federal Register on 1 October by the Department of Defense. Its publication stems from recommendations made four years ago by the Defense Science Board that, if the United States wanted to restrict the export of technologies which might later be used against it, this might be achieved most effectively by shifting the focus of control from end products to the set of "critical technologies" on which the development of these products depended.

To those who might complain that the list is giving away information that could be useful to a potential nuclear power, the department points out that it has not listed many "black arts" or tricks of the trade which cannot be listed as keynote hardware, but may be just as essential to the proper operation or equipment.

The table was compiled in the framework of legislation aimed initially at increasing trade with the Soviet Union and other Eastern European nations: a revised approach was recommended as a way of doing this without sacrificing national security, by placing controls on know-how rather than directly on hardware.

Since then, however, federal policy has been pushed in two other directions. One has resulted from the decision to restrict high technology exports to the USSR following the Soviet occupation of

Afghanistan. The other has been fall-out from the *Progressive* case, where a court agreed that once information has been made publicly available it can no longer be considered classified for legal purposes.

The result has been a schizophrenic desire to tighten restrictions in one direction and loosen them in another. The resulting ambiguity caused an overzealous State Department official earlier this year to prevent a postgraduate scientist studying in the United States from travelling to San Diego for a conference on fusion technology.

David Dickson

European rockets

Range reprieved

The European Space Agency's rocket launching ranges have been reprieved, at least for a further five years. But they will now have to operate on a reduced scale. This was the chief outcome of the meeting last week of the agency's Scientific Programme Committee, at which the representatives of France, West Germany, Norway and Sweden undertook to provide 80 per cent of the cost of operating the two rocket ranges in Sweden and Norway.

The meeting was marked by a further attempt by the United Kingdom to reduce its commitment to the two ranges, at Kiruna (Sweden) and Andoya (Norway), at present estimated to be £1.8 million a year. Although originally a principal partner (with West Germany) to the tune of 40 per cent of the cost, Britain has been reducing its contributions since 1977, largely on the grounds that its need has been reduced since the ending of the Skylark programme.

Last week, the British representatives appear to have startled their partners by suggesting that, from the end of 1980, the British contribution to the total budget of the ranges should be reduced from 20 per cent to roughly three per cent, with a time limit of only three years.

The proposal was not formally accepted at last week's meeting, but will now be put to the Council of the Space Agency for a final determination. Switzerland and the Netherlands also offered last week to continue their contributions at about two per cent of the total, but declined to sign the protocol that would have ensured full operation of the two ranges.

The Science Research Council (SRC)'s position appears to be that its need in the past three or four years has been to launch about six or seven Petrel rockets each year, and that rocket launching facilities in Canada and Alaska can be used on a one-off basis more cheaply than the average cost of the European subscription. Contributing members of the European organization, known as ESRANGE, are required to pay a fee for each rocket launched in addition to their subscription, and neither of these costs includes that of the rocket or of the equipment which it carries.

Judy Redfearn

Table Critical elements in nuclear fuel cycle technology — DoE

	Critical technology	Military use	Civilian use	Foreign availability
A.	Uranium enrichment	Nuclear weapons	Power reactors, research reactors	—
(i)	Gaseous diffusion	—	—	UK, France, USSR, FRG, Netherlands, China, Sweden, Switzerland
(ii)	Gas centrifuge	—	—	USSR, UK, Netherlands, FRG, Japan, France, Pakistan, Australia, Italy, Switzerland, Sweden, Brazil
(iii)	Laser isotope separation	Separation of fissile isotopes	Purifying materials	Canada, France, FRG, Japan, South Africa, UK, USSR
B.	Reprocessing technology	Nuclear material production	Power reactor fuel cycles	Belgium, France, FRG, India, Italy, Japan, Taiwan, UK, USSR
C.	Heavy water production	—	Power reactors	Canada, France, USSR?, Norway?, India, Argentina
D.	Fuel fabrication	Nuclear propulsion, nuclear materials production	Power reactor fuel	France, USSR, Canada, FRG
E.	UF ₆ production	Feed for enrichment plant	Feed for enrichment plant	USSR, France, FRG, Belgium, UK, Japan, China, South Africa, Italy, Canada

British PWR

One year back

The announcement last week by Britain's Central Electricity Generating Board (CEGB) that it will be applying for permission to build its first pressurized water reactor (PWR) at Sizewell in Suffolk was not unexpected. That it may also choose to build another reactor, possibly another PWR, on the same site two to four years later, was more of a surprise. The CEGB says, however, that two 1,200-MW reactors on the site would be roughly in line with permission granted in 1974 to build one 2,000-MW advanced gas-cooled reactor alongside the existing 420-MW Magnox station.

The CEGB will be putting in a formal planning application for the first plant, Sizewell B, early next year. That will include a basic reference design drawn up by the National Nuclear Corporation. By the end of next year, the main stage of the design will be put to the Nuclear Installations Inspectorate for fault analysis, well before the public inquiry expected to take place in the summer of 1982.

The form of the inquiry is not yet settled. Past experience, especially that of the Windscale inquiry in 1978, suggests that, if the board is given the go-ahead, it will be mid-1983 at the earliest before construction can begin, putting back the British government's plans for the next generations of nuclear power stations by one year.

The basic design of the British PWR is that of the Westinghouse Corporation, but its adaptation to local conditions, and especially to British safety standards, is a considerable task. According to the industry, the British standards are among the most stringent anywhere, chiefly because of the multiplicity of stand-by systems required. Designing the buildings to house the Westinghouse system for the soft sands of Sizewell will also add time.

About a dozen of the 200 or so engineers now working on the design have come from Westinghouse and the US Bechtel Corporation, an arrangement that has prompted some opposition from those outside the industry who feel that Britain has the expertise to go it alone. The Bechtel Corporation, which has helped to design PWRs throughout the world, has claimed that the cost of building a PWR in the United States is about \$850 per kW compared with the estimate of £1,000 per kW in Britain. Apart from differences in the accounting basis of these calculations, much of this difference is said to stem from the present strength of sterling, additional safety requirements, construction problems specific to the Sizewell site and the low productivity of the British construction industry. In any case, according to the generating board, the figures now being talked of are estimates, not contract prices.

Judy Redfearn

Mining Polish sulphur Trying harder

Sulphur production in Poland's Tarnobrzeg basin is to be doubled as part of the country's economic recovery plan. The present output of the area is 5 million tonnes per year, one-third of world production, of which 80 per cent is exported, so that this increase could play a major part in paying off Poland's soaring international debts.

Sulphur production is both one of the oldest and one of the newest industries in Poland. In 1415, King Wladyslaw Jagiello granted the miners of Krakow the right to prospect for "sulphurous minerals" in the vicinity. Four hundred years later, in his classic treatise *"On the Resources of the Carpathians and Other Polish Mountains and Plains"* (Warsaw, 1815), the Polish mineralogist Stanislaw Staszic remarked that "Poland stands on sulphur". By the end of the nineteenth century, however, existing mines had been worked out, and in 1921, the last of the old mines, at Posadza, closed down.



Between the Wars, several geologists suggested that sulphur beds might be located in the Miocene sediments of the Sub-Carpathian foreland, but it was not until 1953 that substantial deposits were located at Tarnobrzeg. Open-cast mining began the following year. Towards the end of the 1960s the Gryzbow and Jezioro mines were opened; these use a borehole method of underground melting, and some 80 per cent of Polish sulphur is produced by this technique.

The Polish sulphur industry is now backed by a considerable research and development structure. Environmental problems of the industry and the re-

generation of worked-out pits were elaborated in the early 1970s by a special team at the Krakow Academy of Mining and Metallurgy, and as the officials of the Tarnobrzeg mine point out, land reclamation begins as soon as a particular sector of the pit is exhausted. Plans are in hand to use closed-cycle water in all borehole mines.

Although Poland is the world's largest sulphur producer, there are, however, occasionally odd shortages on the home market. Earlier this year, Gdansk radio noted that the local epidemiological station, which is responsible for environmental monitoring, had been unable to obtain necessary supplies of sulphuric acid. In 1979, it received only 8 litres, instead of the 200 ordered.

The increased production from Tarnobrzeg, which should remedy such defects as well as boosting exports, should not, in the short term, make serious inroads into Poland's sulphur reserves. According to one expert at Tarnobrzeg, at the current rate of production, Polish mines already in operation hold enough reserves for at least another 75 years, and a similar amount of sulphur is estimated to lie in other natural deposits not yet even broached.

In the Soviet Union the outlook for sulphur production seems less optimistic. Although the Soviet Union has some indigenous sulphur, the deposits are deep and difficult to exploit, and some 10-12 per cent of Poland's sulphur exports go to the Soviet Union. A joint Soviet-US survey also indicates that there will be a world shortage in sulphur by the year 2020, because of soaring demands from the Third World. The promotion of research into cost-effective method of recycling sulphur would help the situation — at present, reclaimed sulphur costs four times mined sulphur.

For the moment, however, the main problem faced by the Polish sulphur industry is how to extract the sulphur fast enough. At the Tarnobrzeg open-cast pit, for example, the stockpile between the mine and the processing plant is virtually zero, and a breakdown in the conveyor belt lasting more than a few hours could bring the processing plant to a halt. The transport/export process is also operating full-tilt. Sulphur is exported either by rail or through the liquid-sulphur terminal at Gdansk (the home port of Poland's three liquid-sulphur tankers). So when, early in September as a kind of postscript to the recent industrial unrest in Poland, the Tarnobrzeg sulphur-workers finally went out on strike — seeking pay and social security parity with miners rather than factory workers — the authorities were quick to ask for the election of a strike committee to keep the disruption as brief as possible.

Vera Rich

CORRESPONDENCE

Hurricane damage

SIR — Not only did hurricane Allen select many small island countries, it did so while affecting their capitals the least. Of the nine island countries directly affected by the hurricane before it struck the Central and North American mainland, seven were most seriously affected in areas which did not include the capital city; Barbados, St Vincent, St Lucia, Dominican Republic, Cuba, Jamaica and the Cayman Islands. In nearly all cases, statements of the hurricane's effects issued from the capitals were qualified by references to isolated areas or "the interior" from which reports had not yet been received, but with the expectation that when they were, figures of dead and damage would rise significantly.

When serious hurricanes occur in provincial and rural areas which are normally remote and inaccessible, the degree of local self-reliance and local self-government may determine the capacity for recovery. Dependence on central national government, usually the case, will only function effectively through country-wide communications systems. It is systems of communication, however, which often suffer most significant hurricane damage, quickly isolating provincial and rural areas from the capital, and from each other. Trees fall, blocking roads and causing telephone and electric power cables to be severed, cable poles and radio antennas to collapse and power supplies to fail. Telephone, radio and telex systems become inoperable, and airports close. Roads are destroyed by sea erosion and landslide, and travel by boat is impossible or extremely dangerous until gales have subsided.

The development of international and regional precautions is principally based on improved international communications systems, but effective precautions have to be implemented and undertaken within and by each country. Effective readiness will depend on political will, personal commitment, local understanding and infrastructural resources. Certainly, improvements to inter-island and international communications are needed, but until more efficient national assessments of disaster impact, and consequential needs, are developed through improved national communication of all kinds, there will be little effective use, in disaster, of the improvements to international communications that are being scheduled. More importantly, the present incoming warnings of hurricanes, whilst receivable by governments in the capitals, may not be available to local communities. Ultimately, effective communication facilitates further decision, action or undertaking, which will be the quicker only if administrative and infrastructural resources are upgraded to enable appropriate responses.

Field research from the University of Bath, supported by the Leverhulme Trust, undertaken in two countries (one of them in the Caribbean) has shown the smallest countries to suffer the greatest national scale, as distinct from magnitude, of disaster damage, and hurricane Allen has seemingly selected many Caribbean small countries for reiteration. Provincial districts are more vulnerable to hurricane damage in terms of damage distribution, than the urban centres. Hurricane Allen has not only selected many of

the smallest and poorest countries of the Caribbean, on which to inflict itself, but also the most rural and poorest of those countries. In their experience of hurricane Allen these areas have made manifest a double vulnerability which identifies a need for improved preparedness against such eventualities and greater self-reliance before many more hurricanes have passed.

JAMES LEWIS

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ACHe uses

SIR — We would like to comment on an advertisement from the US Army in the September 11th edition of *Nature* (p. xxxiii) calling for scientists in the genetic engineering field to submit proposals to "clone" the gene for the human enzyme acetylcholinesterase. Isolating the gene for the enzyme and cloning it in bacteria using genetic engineering techniques will allow large quantities of pure acetylcholinesterase to be prepared for the first time. The US Army may have a number of uses for the enzyme in mind: as an antidote to nerve gases for soldiers in battle, as a test for other antidotes and as a test for the effectiveness of new nerve gas weapons. Many workers in the genetic engineering area see the potential for developing this biotechnology into the basis for an ecologically sound and highly energy efficient industry of the future producing useful products. We deplore the signs that the military/industrial powers are moving into the field to distort the technology into the manufacture of commodities of war.

R.G. ELLES, T.R. SKERN, M.E.E. HILL, J.E. ARRAND, D.A. WESTAWAY, C.G.P. MATHEW, A.J. KEELAN, S.M. DARLING, C. COUTELLE, J.M. CRAMPTON, J.T. ROGERS, I.J. JACKSON & R. WILLIAMSON

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Willberforce no ape

SIR — Wrangham's publication (*Nature* 18 September)¹ of the hitherto unknown verses of Wilberforce is interesting and valuable. Unfortunately he repeats the story that Wilberforce played into Huxley's hands, and Huxley scored a great victory. Neither of the two journalists present thought he had. Nor did Joseph Hooker, writing to Darwin the following evening; he thought that Huxley had done well but that it was he, Hooker, who had made the case for darwinism². Lyell thought so too³.

Wilberforce was not an obscurantist. In his review of the *Origins of Species* he was at pains to emphasize that Darwin's theory should be judged only on scientific grounds⁴. Wilberforce allows that Darwin's theory could be true, and "if Mr Darwin can . . . demonstrate to us our fungular descent, we shall dismiss our pride, and avow, with the characteristic humility of philosophy, our unsuspected cousinship with the mushrooms" (ref. 4, p. 231). Wilberforce objected that Darwin had not, in 1860, made out his case, and that there were serious difficulties, in view of the known stability of species, in supposing

that one species could be transmuted into another.

These were serious scientific arguments, worthy of a vice-president of the British Association. Darwin acknowledged their cogency⁵. It was not really until the 1940s that they were adequately answered. Although now we can say with confidence that Darwin was right and Wilberforce wrong, Wilberforce was not being wrong-headed. On the evidence available at the time, his was a perfectly reasonable position to take, and the one taken by most scientists⁶.

It is doubtful that Wilberforce asked Huxley whether he was descended from an ape. It makes a good story, but Wilberforce had used the first person plural in his review, and the use of the first person is borne out by Wilberforce's biography and one — admittedly late — account⁷. What Wilberforce may have asked Huxley in the second person is where he drew the line between human descendants and ape-like ancestors, if, as was generally admitted, the offspring was of the same species as the parents⁸. It is an instance of the Sorites fallacy, of which biologists are impatient, but which has been much discussed by logicians in recent years. Huxley, however, was ready to answer the question he had not been asked. Three months earlier, in the April issue of the *Westminster Review*, he had accused the critics of Darwin of making him out to be no better than an ape himself, and since Wilberforce was now criticizing him for being a darwinian, he must be calling him an ape too.

Wilberforce was, as Wrangham says, both honest enough to see the force of the arguments in favour of evolution and sensitive to some of its implications — not so much theological as human. In 1860 genetic arguments were being adduced in the United States in defence of slavery, and Wilberforce had in his review a witty passage about the colour prejudices of ants, who always have black ants as their slaves (ref. 4, p. 253–254). Evolutionary arguments could be used — in our century have been used — to justify the degradation of man. Wilberforce realized, as these verses show, that a theory's being uncomfortable does not preclude its being true, but that its being true does not prevent its being uncomfortable.

One other moral may be drawn. Science cannot flourish without scientific controversy, and in every scientific controversy most of the views canvassed will ultimately be shown to be wrong. Nobody holds it against Einstein that he confessed himself unable to believe in a dice-playing God. Why is not equal charity extended to Wilberforce's contribution to the British Association?

J.R. LUCAS

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3. Mrs Lyell, *Life of Sir Charles Lyell* Vol. ii, 335 (1881).
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NEWS AND VIEWS

Whatever happened to neutrino mass?

Earlier this year, the high-energy physicists and the rest of us were much excited by preliminary reports that neutrinos may not, after all, be devoid of mass. For a few weeks in the spring, people in several disciplines were more than ready to embark on yet another substantial modification of accepted ideas. Cosmologists and astrophysicists as well as the theoretical particle physicists were involved. If neutrinos have mass, that might suffice to hold the universe together, the cosmologists were saying. Astrophysicists were looking for an explanation of the puzzling discrepancy between the measured and predicted fluxes of neutrinos from the sun. But now, the wave of excitement has died down almost as quickly as it blew up. Observers of the scene may be forgiven for suspecting that the whole affair was a consequence of the notoriously fertile imagination of the particle physicists. What is happening? And what lies ahead?

The events of the past few months are easily narrated. From three independent sources there were reports that neutrinos might be more interesting than the massless (and chargeless) particles that Pauli first postulated (in 1932) to explain why electrons from the beta decay of identified isotopes do not carry away all the energy released in the decay. Most attention centred on reports of conference presentations by Dr F.W. Reines, of the University of California at Irvine, that a reactor experiment had provided evidence of the phenomenon called neutrino oscillation (see Rujula and Glashow, *Nature*, 21 August). In this process, one type of neutrino will spontaneously be converted into a mixture of all types.

When the process was first suggested on theoretical grounds by B. Pontecorvo in 1968, there were of course only two types of neutrinos, corresponding to the electron and the mu-meson. (Strictly speaking, just as there is an anti-electron corresponding to the electron, so there is an anti-neutrino corresponding to the ordinary electron neutrino). Now, with the discovery of the tau-lepton, and the supposition that there must be a corresponding tau-neutrino, there is more scope for oscillation.

Reines' experiment (carried out at the power reactor at Savannah River) is acknowledged to be a difficult one. A reactor on power is a prolific source of neutrinos, but the background of neutrinos generated by nuclear processes elsewhere in the universe is also substantial. The trouble is that the experiment is bedevilled with potential uncertainties and errors — too little is known of the energy spectrum of neutrinos from a power reactor and the frequency of nuclear reactions is small (because neutrinos are what they are). It seems to be necessary to look for a 20 per cent effect (the change in the frequency of the reactions stimulated by the neutrinos) with a piece of equipment of poor precision. Only the accumulation of data over time will provide a clear answer.

This, no doubt, is why Reines's experiment has provoked controversy among the particle physicists, most of it well-mannered. Some complain at the statistical treatment of the data, others at the physical interpretation of the observations. All, even Reines, appear to agree that more data (and therefore time) is needed before anybody can be certain. This is why many people see more hope in experiments which are quite different. Some of the difficulties may be overcome by working with energetic beams of neutrinos produced artificially by particle accelerators.

Others, however, think there may be more future in experiments designed to measure directly the mass of the electron

neutrino (if, indeed, it should differ from zero). Oscillation and mass are connected by the theories now being designed to unify three of the four natural forces of nature (gravity excluded). If neutrino oscillation, or interconversion of neutrinos takes place, then neutrinos should also have mass — and each of the three types of neutrino should have a different mass. The demonstration of either non-zero mass or of neutrino oscillation would most probably bring comfort to the theorists.

Attempts at the direct measurement of neutrino mass are by no means novel — if, in the old days, the theorists went around saying that neutrinos have no mass, what more natural than that experimentalists should set out to check the assertion. Moreover, there seems very little doubt that the best route to a direct measurement of the neutrino mass is by observation of the electrons emitted by radioactive tritium, one of the least energetic of beta decays and thus one in which the energy-spectrum of the decay electrons will be most sensitive to the mass of the neutrino.

In the late 1960s, measurements like these had set an upper limit to the neutrino mass of 60 electron-volts, itself something like one part in 100,000 of the mass of the electron. More recently, however, a group from the Institute of Theoretical and Experimental Physics in Moscow has announced that measurements of the energy spectrum of decay electrons from tritium gives a mass for the electron-neutrino which is different from zero and somewhere between 14 and 40 electron-volts.

So far, the chief effect of this has been to stimulate others to embark on similar investigations and several tritium experiments are under way. (For example, in Stockholm and in Chalk River, Canada — and no doubt in many other laboratories.) The experimental difficulties are almost bizarre.

The objective is to measure the energy of decay electrons accurately when the energy is a fraction of an electron-volt — and the accuracy that matters most is that when the energy is least and where, in the nature of beta-decay, the number of decay electrons is also least. Tritium must be mounted in such a way that decay does not itself disturb the measurements — in the Moscow experiment, for example, tritium was incorporated into sugar molecules whose disruption after a decay was even in itself a source of experimental confusion. Evidently, the smaller the neutrino mass, the more difficult these experiments will be.

If the upper limit on the neutrino mass gets too small, the cosmologists will of course lose interest. They require a mass of some 30 electron-volts to close the universe. Nothing less will interest them. For accounting for the unexpectedly small neutrino flux from the sun, the mere phenomenon of oscillation will suffice. The mass is comparatively unimportant.

The particle physicists are the most downcast by the cloud now hanging over the non-zero neutrino mass. Their problem is simple. If the unified theories of particles are to work as simpler but apparently successful theories have worked in the past, neutrino oscillations should happen, and the neutrino mass should differ from zero, if only by a very small amount. In other words, there is a now familiar dilemma. A favoured theory predicts some phenomenon whose measurement is inherently difficult. Gravitational waves predicted by the general theory of relativity are an obvious illustration. But these are precisely the circumstances in which, with the best will in the world, people are tempted to make more of their data than is fair.

Nations and numbers, 1980

from Robert M. May

THE continuing increase in the global population is such that most of us have difficulty keeping track of the current figure — for 1980 it is 4.4 billion. As noted in previous News and Views articles²⁻⁵, this global number conceals much variation among individual nation states and among regional groupings. Any analysis of population trends and prospects must deal with these variations; a particularly good recent analysis is by Mauldin⁶ (in the special 'Centennial Issue' of *Science*). Over the 30-year span 1950-1980, the population of the world has grown by 70%, from 2.5 billion to 4.4 billion. In this period, Europe underwent a relatively small increase of 23%, the USA and the USSR both had increases of just under 50%, while the developing countries almost doubled their population (from 1.7 billion to 3.3 billion, with increases of 125% in Latin America, 114% in Africa and 85% in Asia).

Looking ahead, Mauldin⁶ suggests "the prospects until the year 2000 are for a slowing of growth rates for all areas except Africa, but the numbers to be added will be enormous". The United Nations projections foresee continued decline in the rate of population growth in developed countries; the estimates are for an increase of less than 8% over the next 20 years in Europe, and about 17% in the USA and the USSR, to give an average figure around 12-13% for all developed countries. The

so-called developing countries seem likely to increase their population by about 50%, broken down as 76% in Africa, 65% in Latin America, 55% in South Asia and about 24% in East Asia.

All this adds up to a remarkable change in global demographic patterns over the second half of the twentieth century; over this 50-year span the developed countries are likely to have increased their populations by about one-half (from 830 million to 1270 million), while the developing countries are likely to have seen a threefold increase in population (from around 1.7 billion to around 4.9 billion).

These facts and projections are summarized in Figure 1, which shows the age distribution of the total populations of developed and of developing countries in 1980, and the corresponding UN projections for the year 2000. The high proportion of young age classes in developing countries is characteristic of relatively rapidly growing populations. Even though growth rates are likely to slow down over the next 20 years, the large number of people yet to move into their reproductive years in developing countries means that populations will continue to grow. This phenomenon — the "momentum of population growth" — can be expressed most dramatically by noting that even if zero population growth (ZPG — birth rates equal to death rates)

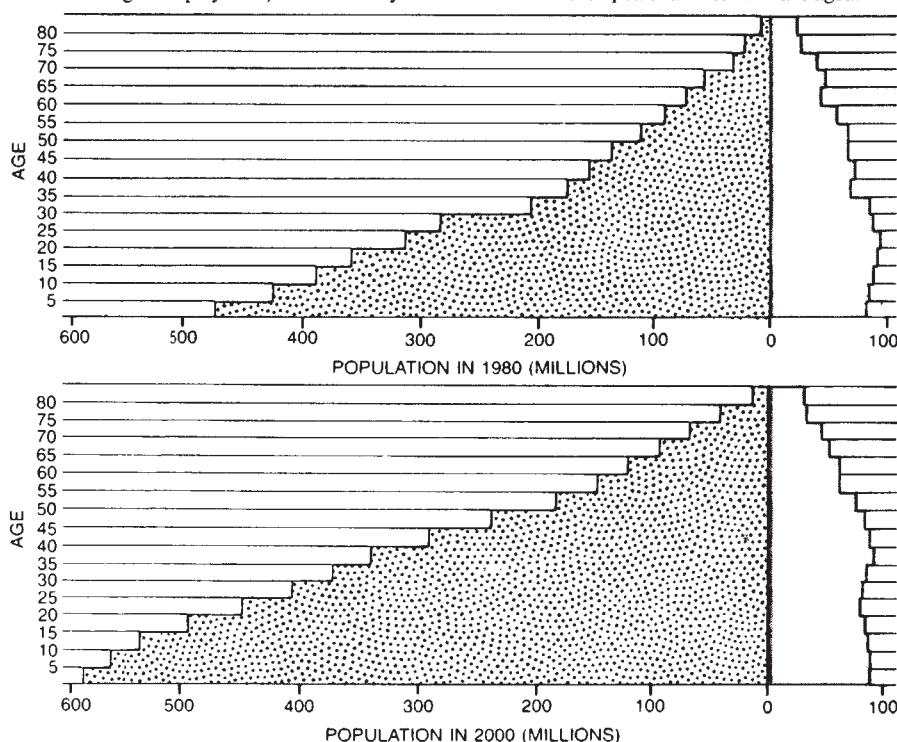
could be attained overnight in all developing countries, they would still be committed to a rough doubling of total population before coming to equilibrium; the triangular age profiles on the left in Figure 1 will eventually settle to the more rectangular pattern of the age profiles on the right (characteristic of ZPG populations), but these 'rectangles' must be erected on the large base of the infant cohort.

Observations about the size and growth of the world's population are notoriously subject to many uncertainties⁶. As discussed previously³, the current population of China, which represents about one-quarter of the global total, is unknown to within 5% or arguably even 10%. The Chinese census planned for 1981 may produce a firmer figure. The most populous African country, Nigeria, is estimated as somewhere in the range 70 to 85 million, but it could be more. The current fuss about the 'undercount' of city populations in the 1980 USA census, and its consequences for Federal aid distributed according to population-based formulae, emphasizes that even the US population is uncertain to within about 2%. As we look to the future, we peer through a glass ever more darkly.

With these caveats, the projections summarized above are based on country-by-country extrapolations of present trends⁶⁻⁸. These vary greatly among the developing countries, as can be seen from Table 1. The Table shows the crude birth rates (expressed as births per 1,000 of population) in 1965 and in 1975, for the less developed countries with populations in excess of 35 million (Vietnam is excluded because the information about vital rates is thought to be unreliable). The Table also shows the percentage change manifested in the crude birth rate between 1965 and 1975 (with an asterisk denoting no significant change), along with a qualitative assessment of the strength of family planning programs in the country.

Table 2 represents a more encompassing, but less detailed, version of Table 1. This Table shows the average percentage decline in crude birth rates between 1965 and 1975 in 94 developing countries, grouped according to social setting and the intensity of family planning efforts. Social setting is a variable which combines rankings on 7 socioeconomic scales: adult literacy; school enrollment; life expectancy at birth; infant mortality rate; per capita gross

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national product; percentage of males employed outside agriculture; and percentage of population living in cities of 100,000 or more^{6,9}. Although there continues to be much dispute and uncertainty about the cause of recent fertility declines in developing countries, Table 2 shows that both 'social setting' or degree of development and family planning programs have significant correlation with patterns of declining fertility⁹.

Most population estimates for the year 2000 cluster around 6 billion (the 'high, medium and low' UN estimates⁸ are 5.9, 6.2 and 6.5 billion), mainly set by the 'momentum' effects discussed above. Any estimate of the eventual steady state global population is necessarily much more uncertain. The momentum of population growth means that populations in developing countries will continue to undergo significant increases until well into the twenty-first century, and there is no consensus as to when, and at what rate, fertility will begin to decline in Black Africa, Bangladesh and Pakistan. Mauldin's⁶ assessment is that "if the leaders of the major developing countries view rapid population growth as an impediment to economic development and act accordingly, and if the donor community also continues and perhaps increases assistance to such countries, the population of the world might plateau around 10 billion. But larger numbers are all too possible." And none of the estimates take any explicit account of the problems of feeding these multitudes. □

Table 1: Decline in crude birth rates, 1965-1975, in countries with populations of 35 million or more (after Mauldin⁶).

Country	1980 population (millions)	crude birth rate (per thousand)		percentage decline in crude birth rate, 1965-1975	family planning program
		1965	1975		
China	975	34	22	35	strong
India	694	43	36	16	moderate
Indonesia	152	46	36	22	moderate
Brazil	122	42	33	21	weak
Bangladesh	89	48	48	*	weak
Pakistan	82	47	47	*	weak
Nigeria	77	49	49	*	weak
Mexico	70	44	37	16	weak
Philippines	51	44	34	23	moderate
Thailand	48	44	33	26	moderate
Turkey	45	40	34	16	weak
Egypt	42	41	37	10	weak
Iran	38	45	45	*	moderate
South Korea	38	32	22	29	strong
Burma	35	40	40	*	none
Total	2,523	40	33	20	

Table 2: The average decline in crude birth rates between 1965 and 1975 (expressed as a percentage) is shown for 94 developing countries, grouped according to social setting and the intensity of family planning efforts. The numbers in brackets indicate the number of countries in each particular grouping. (Compiled from data summarized by Mauldin and Berelson⁹.)

	family planning effort				
	high	upper middle	lower middle	low middle	Total
high	30 (9)	29 (5)	9 (4)	* (6)	19 (24)
upper middle	24 (1)	18 (8)	6 (11)	* 4	10 (24)
lower middle	23 (1)	14 (2)	* (8)	* (12)	* (23)
low middle	— (0)	— (0)	* (7)	* (16)	* (23)
Total	29 (11)	21 (15)	* (30)	* (38)	9 (94)

*denotes no significant change

Towards the conquest of hepatitis B

from Arie J. Zuckerman

THE current issue of the *New England Journal of Medicine* (9 October) reports the remarkable success of a carefully controlled trial of hepatitis B vaccine in a high risk population in the United States. This achievement by Wolf Szmuness and his colleagues must be regarded as a milestone in the annals of preventive medicine because hepatitis B virus has not yet been propagated and serially passaged in cell culture. The credit for the development of this unusual vaccine lies with the series of studies conducted between 1970 and 1973 with the MS-2 strain of hepatitis B by Krugman and his colleagues. (*J. Infect Dis.* 122, 432; 1970; *New Engl. J. Med.* 288, 755; 1973).

Their studies demonstrated that diluted serum containing hepatitis B virus heated to 98°C for one minute prevented or modified infection in 69 percent of susceptible

individuals. These observations provided the stimulus for the preparation of a vaccine using the 22nm small spherical hepatitis B surface antigen particles purified from the plasma of healthy carriers and inactivated with formaldehyde, with or without treatment by heat.

Hepatitis B is of tremendous importance in every field of medical practice; with progression of the infection some patients experience chronic liver disease including cirrhosis, and there is also an association with primary liver cancer. Infection with hepatitis B virus, particularly in early life, may be followed by the persistent carrier state. The world reservoir of carriers at present is estimated to number about 200 million, and they too may suffer liver damage.

The wide range of parenteral and inapparent parenteral routes of transmission of hepatitis B includes the transfusion of blood and certain plasma derivatives, the use of inadequately sterilised syringes, needles and instruments, sexual contact, particularly among promiscuous homosexual men, and

possibly transmission by blood-sucking arthropods. In some regions of the world, transmission of hepatitis B to infants may be as high as 50 percent from carrier mothers, or from mothers with acute infection in pregnancy or shortly after delivery.

A vaccine against hepatitis B is urgently required for groups at high risk of infection. These groups include patients requiring multiple transfusions, patients with natural or acquired immune deficiency and patients with malignant diseases; patients and staff of haemodialysis, transplant and oncology units and residents and staff of institutions for the mentally-handicapped. Viral hepatitis is an occupational hazard amongst health care and laboratory personnel. High rates of infection occur in homosexual men, drug addicts and prostitutes. The high rates of perinatal transmission of hepatitis B in some regions may make protective immunisation of susceptible women of child-bearing age and of infants the only practical way of interrupting transmission of the infection. Immunisation must also be considered for

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persons living in areas where the prevalence of infection is high.

Szmunn *et al.* chose for the trial a young, healthy population of male homosexuals who are known to be at an unusually high risk of infection (*Ann. intern. Med.* **83**, 489; 1975). The cooperation of the participants in this meticulously organised, placebo-controlled, randomised, double-blind clinical trial of the hepatitis B vaccine was excellent: the loss was 15.4 percent of those entered, a rate much lower than the projected 40 percent annual rate of loss. Of the 1,083 susceptible individuals who received the first injection, 96.5 percent received the second injection six months after the primary inoculation. The follow-up was excellent and of the 6,332 scheduled visits and bleedings, 91.2 percent were actually carried out.

Hepatitis B surface antibody developed within one month of the injection in 31.4 percent of recipients of the vaccine; this increased to 77 percent one month after the second injection, to 87 percent within three months and to 90 percent within six months. The booster injection at six months produced a further increase to 96 percent and high titres of antibody persisted, essentially unchanged, for 18 months of follow-up in 65–70 percent of

the subjects. There is the possibility, of course, that the vigorous antibody response found in some of the subjects was boosted naturally by repeated exposure to the virus. Nonetheless, the antibody response resulted in a dramatic reduction in the incidence of infection in the vaccinated group. This high protective efficacy was observed in all sub-groups of participants, including those with a large number of different sexual partners who are most at risk.

Much has been written about the possible undesirable side-effects of a vaccine prepared from plasma of persons who are persistent carriers of hepatitis B surface antigen (Zuckerman, *Nature*, **255**, 104; 1975) and guidelines have been proposed by the World Health Organisation for the preparation of such hepatitis B vaccines (WHO *Techn. Rep. Series*, **602**; 1977). Rigorous purification of the 22nm spherical hepatitis B surface antigen particles was undertaken during the preparation of the vaccines used in the trial by Szmunn *et al.* Digestion with pepsin ensured freedom from contaminating host substances, and the purified particles were treated with formaldehyde to inactivate any residual live virus(es). The vaccine was

tested for live hepatitis A virus in susceptible marmosets and for live hepatitis B virus in susceptible chimpanzees and it was also tested extensively to ensure the absence of extraneous viruses. There was no evidence that residual hepatitis B virus was present in the preparation and no seroconversion in the vaccinees could be attributed to the intact virus. It is gratifying that most of the adverse reactions were relatively minor, rarely more than a sore arm, although rashes, nausea, vomiting, joint pain and fatigue were also reported. Fever occurred in 2.6 percent of those vaccinated and in 2.2 percent of the those receiving the placebo and, indeed, the incidence of all side-effects was similar in the two groups.

Finally, a most important finding was that the development of hepatitis B surface antibody is synonymous with protection against hepatitis B. Therefore, as the authors point out, once immunogenicity has been demonstrated in a particular population which might not respond as well as young, healthy adults, there should be no need to prove efficacy again; indeed such a procedure might be ethically questionable and delay the availability of the vaccine to those who need protection.

Variable stars and galaxies

from Julian Osborne and Richard Cole

The night sky may appear almost unchanging but a closer look reveals that there are many astronomical objects showing some form of variability. The extent and form of this variability was the topic of a recent conference* and, at its close, one might have been forgiven for thinking that there could be no safe standard stars left!

Variability seen in the whole Sun was reviewed by Stenflo (Institut für Astronomie, Zurich). Of the short timescale variations, only the 5 minute oscillations are well established. Diameter variations with periods between 5 minutes and 1 hour are still controversial and theoretical understanding difficult because of the large number of eigenmodes with periods in this range. Exciting news came with the confirmation of the 160.01 minute period by Fossat and Grec (University of Nice). The period has been seen as a Doppler pulsation since 1974, and although observed by groups in Crimea, Birmingham and Stanford, has remained doubtful because 160.01 minutes is close to one-ninth of 24 hours. The University of Nice group went to the South Pole and obtained an uninterrupted run of data from the full disk lasting 120 hours.

Observations from the South Pole both suppress Earth rotation effects, and make use of the very good sky transparency. All groups report the same period, amplitude

and phase but a theoretical explanation awaits identification of the order and mode of the oscillation. Kotov (Crimea Astrophysical Observatory) reported that the amplitude changes at the rotation period of the Sun and that there are small accompanying variations in brightness and cm radio emission.

Very long period variations were reported by Hughes (Queen's University, Ontario; Sterrewacht, Leiden) who was analysing 32 years worth of 10 cm radio data. He noted periodicities at 1,150 days and about 700 days, neither of which were present throughout the data. A 750 day periodicity was seen during 1970–75, as claimed by Sakurai (*Nature* **278**, 146; 1979) for the neutrino flux, and as seen by Shapiro *et al.* (*J. atmos. Sci.* **19**, 506; 1962) in the relative sunspot number. Of course, a relationship between the solar core and the overall activity of the sun would be very interesting. Other periodicities have been seen, all of which are consistent with a 260 day variation.

Ciatti (University of Padua) reviewed our knowledge of the 135 classified supernovae, showing the clear distinction between type I (64%, 3 — 4 $M_{\odot}$, M-S progenitors from old disk population) and type II supernovae (28%, progenitors greater than 10 $M_{\odot}$, stars in spiral arms). Hughes reported the discovery of a new SNR (G109.2-1.0) with the Westerbork telescope. The remnant has a good circular shell of 24 arcmin diameter, and is at a distance of 4.7 kpc. It has been seen as an

X-ray source by the Einstein satellite.

The 1979 Type II Supernova in M100 was exceptionally well observed, there being data in the radio, IR, optical, UV and X-ray ranges. Evans (University of Keele) showed that only pre-existing circumstellar graphite grains explained the infrared excess seen 260 days after maximum light.

Palumbo (Istituto TESRE del CNR, Bologna) reported UV observations which show the existence of an additional slow moving shell (10^3 km s^{-1}) exterior to the expanding photosphere (10^4 km s^{-1}). The shell, which had twice the radius of the photosphere at one week, is thought to be due to the progenitor's stellar wind, which is required to be $10^{-4} M_{\odot} \text{ yr}^{-1}$ to provide the 0.2 $M_{\odot}$ of the shell. Whilst not seen at radio wavelengths 10 days after maximum, it was observed one year later ($S_{6\text{cm}} = 5 \text{ mJy}$). So far there is only an upper limit to the X-ray emission. Double structure has also been observed in the Crab Nebula by Danziger (ESO), in that there appears to be two expansion velocities for each spectral line ($\Delta V \sim 265 \text{ km s}^{-1}$).

In a review on pulsars, Lyne (Jodrell Bank) said that the recent Molonglo survey had increased the number of radio pulsars to over 320, including 3 binary pulsars (of which only 1913 + 16, shows energy loss attributable to gravitational radiation). There are thought to be at least 5×10^5 pulsars in the Galaxy. (Taylor, *8th Texas Symp.* 1977 and Davies *et al.* *MNRAS* **179**,

*Fifth European Regional Meeting of the IAU — "Variability in stars and galaxies" was held at the University of Liege, Belgium, on 28 July 1980.

635, 1977). These have an average lifetime of 4×10^6 years, derived from their characteristic age and migration velocity. Together, these give a pulsar creation rate of about 1 every 8 years, greater than the supernova rate of 1 every 20 to 100 years. Thus, there appears to be more pulsars created than supernovae, although no other birth mechanism for pulsars is known which would provide the high migration velocity (150 km s^{-1}) and scale height (400 pc). The problem would be relieved if the Galactic electron density was 0.02 cm^3 instead of 0.03 cm^3 , or if pulsars could be created in supernovae that did not create optically visible remnants. However, Heffland's work with the Einstein satellite (*IAU Symp. 95*, Bonn Aug. 1980) shows that many young supernova remnants do not contain pulsars, thus making the problem worse.

Van Paradijs (University of Amsterdam, MIT) provided a lucid review of X-ray bursters. Dividing the binary galactic X-ray sources according to the ratio of their X-ray and optical luminosities gives two distinct types of X-ray emitters: (1) L_x/L_{opt} less than 10; primaries are O, B stars $M_v \sim -5$, hard X-ray spectra, X-ray pulsars, X-ray eclipses common. (2) L_x/L_{opt} greater than 10, primaries are 'Sco type', $M_v \sim +3$, soft X-ray spectra, bursters.

A typical burst will have a rise time of 1 second and a decay time of 10–60 seconds. There are, however, two types of burst activity. Type I bursts are seen from all bursters, they occur every couple of hours and their spectra soften during decay. Type II bursts are only seen from the 'Rapid Burster' (MXB 1730-335), which also exhibits type I burst activity. The type II bursts occur about every 10 seconds and have spectra which do not soften. The energy of a type II burst is proportional to the time from the previous burst, implying accretion build up and overflow.

The estimate of $1-5 M_\odot$ for the mass of the X-ray emitter and the estimated radius of the emission region (10 km) have given rise to a successful model for type I bursts. In this, the X-ray emission is due to thermo-nuclear flashes in the helium rich material on the surface of a neutron star, this being accreted from a binary companion.

This model describes the bursts well, with the bursts ceasing if the accretion rate is so great as to cause continuous nuclear burning. Until recently there was no evidence for the binary nature of the bursters but, recent spectral observations of the optical counterparts suggest a companion to CenX-4 (*IAU circ.* 3487), and an accretion disk in AO620-00 (Murdin *et al.* *MNRAS* 192, 709; 1980). The optical counterparts of these stars, and of A1742-28, are K type stars.

Correlated optical bursts have been seen in three sources so far. (1735-44, 1837+05, 1636-53; Van Paradijs). The energy of the optical bursts is 5 orders of magnitude

lower than that of the X-ray bursts and are thought to be due to reprocessing of the X-ray flux. There is a 2 second delay between the X-ray and the optical bursts, reprocessing might cause a delay of 0.5s so the implied distance to the reprocessing site is 1.5 light seconds. It is expected that the reprocessing occurs around the edge of an accretion disk, the size of which implies a neutron star companion of $0.4 M_\odot$ consistent with K-type stars.

Pederson (ESO) reported optical and X-ray coverage, noting one slow burst (rise time 3-4 sec) in optical and X-ray from MXB 1636-53, and one fast optical burst (rise time less than 200 msec) from MXB 1735-44, for which there was no X-ray coverage. Infra-red bursts from the 'Rapid Burster' were described by Jones (Imperial College). This group has discovered very bright bursts ($m_k \sim m_h \sim 6$) of ~ 10 sec duration with fast flashes (some less than 0.3 sec) superimposed. These bursts are only an order of magnitude less intense than the X-ray bursts, but do not follow the known type I or type II behaviour.

Walker (RGO) has been studying the blue light curve of the black hole candidate Cyg X-1 over the past 8 years. He has found that the light curve and period have varied. A periodic change is suggested, such as a third object (period > 18 years) or precession of either of the known components.

His most recent data showed a dramatic change in the light curve. The primary maximum had decreased and the secondary maximum had increased and attained a double structure. This behaviour had been seen once previously, just before an X-ray transition. Evidently, an X-ray transition did take place between mid May and early June of this year (*IAU Circ.* 3491).

Shklovsky (Space Research Institute, Moscow) described his model of the

peculiar object SS433, as an O, B super giant with an extreme stellar wind ($10^{-4} \text{ m yr}^{-1}$) in a binary system with a neutron star of low magnetic field at the centre of an accretion disk. These are surrounded by an expanding cloud, optically thick to infrared radiation. The moving lines are reproduced by blobs which become optically thin to $H\alpha$ as they are blown from the accretion disk onto the circum-system cloud. These blobs become shocked and heated to 10^{7-8} K to produce X-rays.

King (University of Leicester) confirmed a previous X-ray iron line detection, and suggested a change of the X-ray continuum; no periods were found in the range 1-200 days, despite considerable X-ray variability. The infra-red flux is also variable ($\Delta M = 1.6$), the IR colours however, remain constant. There is a suggestion of an 11.8 day IR period, but no period was seen at 13 days.

Observations of SS433 reported by Schilizzi (Dwingeloo Radio Observatory, Holland) showed milli arcsec variation within 6 months. At a larger scale, Spencer (Jodrell Bank) described the radio structures as consisting of an unresolved central core (less than $0.3''$) with a flat spectrum and a $3''$ halo with a spectral index of -1.1 , elongated in the direction of the SNR W50. He noted a 1 week flare in the core which was not seen in the halo.

Ciatti has undertaken a study of moving lines, observing that they vary irregularly in intensity and wavelength, sometimes forming multiple systems. He notes that the velocity and angle of the jets varies night by night whilst following the general precession. His best fit period is 165.5 days, using 200 nights observations over 3 cycles. The intrinsic colours of SS433 lead Firmani (Instituto de Astronomica, Mexico) to conclude that the primary is an early type star intermediate between Of and WN8. These stars are of $15 M_\odot$ burning helium in

100 years ago PHYSICS WITHOUT APPARATUS



To illustrate the geometrical laws of refraction through lenses, a good reading-glass of large size is a desirable acquisition. Spectacle-lenses, though of smaller size, and therefore admitting less light, are also of service. In the absence of any of these

From *Nature* 22, 7 October, 541 & 537, 1880.

articles, it is generally possible to fall back upon a water-decanter, provided one can be found of a good globular form, and not spoiled for optical purposes by having ornamental work cut upon the sides of the globe. The figure shows how this decanter, filled with water, is to be employed. It is held a few inches away from a white wall, and a candle is placed at the opposite side, so that its light falls through the decanter on to the wall. The candle is moved towards or away from the decanter until the position is found in which its rays focus themselves upon the wall giving a clear inverted image of the candle flame upon the wall. The experiment may be varied by setting down the candle on the table and then moving the decanter to and fro until a definite image is obtained. If a large hand reading-glass be available, the image will be much clearer than with the improvised water-lens; and a further improvement in the manner of experimenting may be made by using a screen of white paper or card instead of a whitened wall on which to receive the image.

their core, with $2 M_{\odot}$ H envelopes. The time to loose this envelope gives a 20,000 years life time for the SS433 phenomenon. He expects there to be 10 SS433 type systems in the galaxy.

Pauliny-Toth (Max-Planck-Institut) presented interesting pictures of the variations in the milliarcsec structure of active galactic nuclei. He mentioned 3C84, in which 3 bright regions are expanding but not moving apart, the rate of expansion implies that the source 'began' 20 years ago; all regions having the same spectrum. He discussed 4 sources showing apparent

super-luminal velocities: 3C273, in which 3 central compact components are separating in the direction of the main jet; 3C345, in which a double component has changed to a core-jet structure; 3C120, in which there were strong outbursts in 1971-74 when the source was double (the central source is now a core-jet and there are no outbursts); and 3C279, which is showing 21c separation of a double source, and 40c separation of a new third component. By contrast, NGC 1275 shows only a slow expansion (0.1c) and no separation of the components. □

On the Tibetan plateau

from Peter Jackson

THE Tibetan Plateau has long fascinated not only romantics and mystics, but also geoscientists. It has been recognised as one of the most interesting areas of the globe particularly in relation to continental drift and tectonics, since it was here that the Indian plate, which broke away from Gondwanaland, crashed into the Eurasian plate about 38 million years ago. The suture line lies along the valley of the Tsangpo river, about 100 kilometres north of the main Himalayan axis.

The area remained generally closed to science although, after the Peoples Republic of China asserted itself in the area in the 1950s, reports filtered through of some activity by Chinese scientists. It was thus with some excitement that geologists and natural scientists received invitations from Academia Sinica to participate in a symposium on the geology and natural history of the Qinghai-Xizang (Tibetan) Plateau. Over 60 scientists from 17 countries gathered in Peking in May for a week of meetings at which the Chinese reported in detail on the results of 20 years of research in Tibet.

Liu Tung-shen, Vice-Chairman of Academia Sinica, told the meeting that the rich fossil beds on the plateau had yielded 3,800 species of over 30 families in recent years, compared with only 300 before 1950. In the Himalaya, fossils of *Glossopteris* and cold water biota were found in late Palaeozoic beds, while north of the Tsangpo fossils of *Gigantonoclea* and warm water biota were uncovered in the Permian formation, indicating the importance of the valley as a palaeontological boundary.

Palaeogeomagnetic study confirmed that areas to the north and south of the river valley belonged to different plates — to the north the crust is about 70 kilometres thick, and to the south about 50 kilometres thick. Isostatic anomaly in the Everest area is +120mg, and uplift is continuing as the Indian plate still pushes north. For the last 100,000 years since the Pleistocene a rise of

10 mm per yr has been estimated by Chinese scientists.

It is believed that the Himalayan system may have been completed between Miocene and Pliocene, but even in late Tertiary the plateau may not have been more than 1,000 metres in elevation, judging from fossils of the three-toed horses *Hipparion* found during the recent explorations. The Himalayas are considered to have reached great heights in the late Pleistocene, bringing about a climatic change to present conditions when they blocked the warm wet monsoon and made Tibet a cold, arid area.

The week in Peking was an absorbing and active one but was just a preliminary to the real object of interest — a two-week field excursion to Tibet, from Lhasa westwards and finally passing through the Himalayas to Kathmandu.

The high altitude physiologists found that the Tibetans, although their capital Lhasa is at a height of 3,660m, had the same blood haemoglobin values as lowlanders in, for example, Shanghai, but lowlanders up on the plateau showed a significant increase in haematological values, which became normal only after 10 to 15 years. The surprising revelation was that when lowlanders, whose haematological values had become normal, returned to the lowlands they suffered a period of one to three months of re-adjustment. A high percentage of the lowlanders have to leave the plateau because, after a year or two, they develop chronic mountain sickness with a tremendous increase in red blood cells.

Many of the visiting scientists had taken with them NASA earth satellite imagery of the area through which we passed. Glaciologists noted that studies of the imagery in the United States tied in well with what was actually observed. The surprise in paraglacial geology was that typical phenomena such as the sorting of stones by freezing and thawing on slopes was not as widespread as expected at such high altitudes. It became evident that this was because of the extreme dryness of the air so

frost action was almost absent. Characteristic phenomena were found only in moist areas.

Glaciation in Tibet appears to have been little more during the Pleistocene than it is today, and E. Reiter of Colorado State University suggested that this was because the Indian summer monsoon, which brings in moisture and snow, was either very short during the Pleistocene or sometimes even absent. He suggested, on seeing heat shimmer in temperatures of about 9°C on a 5,220 metre pass, that heat from the plateau generates an anti-cyclone and draws in the monsoon from India. During global cold periods, however, such as during the Pleistocene, when the gradient between the equator and the poles is strong and the sub-tropical high pressure belt is drawn towards the equator, the northward pull of the Tibetan plateau may be insufficient to bring in the monsoon.

Most of the valleys had sand dunes, which, in some cases, such as around Lhasa, were shapeless conglomerations resulting from complicated wind patterns. But along the Tsangpo near Xigatse, and further west near Tingri, sickle-shaped dunes were seen, which were the result of a steady up-valley wind. T. Pewe, of the University of Arizona, remarked that silt particles from among the sand had blown up the hills and walked back down forming areas of retransported loess favourable to agriculture. He said that according to Professor Liu, this was the first report of loess in the area.

Scientists of several disciplines commented on signs of over-exploitation and damage to the physical environment, which indicated the need for considerable care in the future economic development of Tibet. Almost every other cart met on the roads carried fuel. Men and women were seen trudging homeward with heavy loads of shrubs, such as *Sophora moorcroftiana*, which had been dragged out by the roots.

Jack Ives, of the University of Colorado, pointed out the conspicuous and recent gulleying and dissection of fans resulting from deforestation and over-grazing, probably combined with the development of a colder and more arid climate in the last 3,000 years. Ives had presented a paper at the symposium on mapping natural hazards, which stressed the dangers of placing settlements on alluvial fans because of floods and rock and mud flows. In Tibet many villages were placed just there, presumably because of the availability of water. It was agreed by both foreign and Chinese scientists that there was a need for collaborative research to pinpoint dangerous trends, to help decision makers improve the welfare of the people of Tibet and to achieve a better ecological balance and effective management of natural resources. It was considered especially important to use new techniques developed in the US for areas where the data base is short — as it is in Tibet — to predict floods and other dangers to settlements on alluvial

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fans.

While Chinese experts pointed to some remarkable results in agricultural production and spoke of the potential of Tibet, the visitors stressed the need for caution in view of the short growing season, which could be seriously affected by variability in annual rains, possibly resulting in bad harvests and famine.

Perhaps the least satisfied of the visitors were the zoologists, despite the thrill of collecting lizards, fish, butterflies and small fauna at record altitudes. Brought up on old records of remarkably tame wildlife in Tibet — wild yak, wild ass, blue sheep, antelope, gazelle, geese and duck — they were disappointed to see no large mammals, and birds were generally sparse in

numbers. There were only two or three sightings of shy bar-headed geese (*Anser indicus*), which used to be numerous and confiding on the plateau. This seemed to indicate strong hunting pressure in contrast to the former reverence for wildlife. However, Chinese zoologists reported that there were still populations of most Tibetan species on the sparsely inhabited central plateau, which whetted appetites for further studies.

Collaboration between Chinese and foreign scientists has already begun, and, as a result of the symposium, there is now likely to be a burst of activity aimed at unravelling the secrets of Tibet and applying the knowledge there and elsewhere. □

Mycorrhiza and crop production

from D.S. Hayman

VESICULAR-ARBUSCULAR mycorrhiza, a symbiotic fungus-root association, can greatly increase the utilization of phosphate present in soil. Recent research suggests that the symbiotic association can be harnessed to achieve more economical use of costly superphosphate fertilizers and better exploitation of cheaper, less soluble rock phosphate.

The vesicular-arbuscular (VA) mycorrhizal fungi infect more plants than any other fungi and are necessary for many plants to grow well, but until recently, they had escaped widespread attention. This is for two reasons: first, the association is so well balanced that young feeder roots show no sign of damage even when densely infected, and second, the fungi themselves cannot as yet be cultured in the absence of a living root and so do not appear on agar plates used to isolate other soil and root-inhabiting micro-organisms.

VA mycorrhizal infections are seen as vesicles and arbuscules borne on the mainly aseptate hyphae occupying the root cortex. Similar structures have been reported in fossil roots some 300 million years old and are present in their modern relatives (ferns, psilopsids and clubmosses), as well as in some gymnosperms and angiosperms colonizing most habitats around the world.

VA mycorrhiza is found in most agronomic species except the brassicas and sugar beet and is widespread in the two major crop families, Leguminosae and Gramineae. Important crops where benefits from mycorrhiza have been shown include pasture and forage legumes (but not lupins), maize, wheat and barley, potatoes, many vegetables, herbaceous and tree fruits, and many tropical plantation crops. Temperate zone plants with thick fleshy roots and few, short root

hairs generally benefit considerably from VA mycorrhiza; for example, onions, citrus, grapevine, sweetgum and *Coprosma*. In the tropics, pineapple, *Stylosanthes*, *Centrosema*, coffee, tea, cocoa, oil palm, papaya and rubber all have dense VA infections. Cassava appears to be obligately mycorrhizal which probably explains why it is able to grow in impoverished phosphate-deficient soils (Van der Zaag *et al.*, *Field Crops Res.*, 2, 253; 1979) while needing high phosphate concentrations in solution culture.

The extent to which particular plants depend on mycorrhiza is largely governed by their root morphology. Generally those with the most limited root-soil contact (coarse, hairless roots) benefit most from the network of mycorrhizal hyphae extending from the mycelium inside the root to the surrounding soil. Ions such as phosphate are fairly immobile and a phosphate-depletion zone often develops around roots in phosphate deficient soils. The hyphae are able to reach beyond this zone and directly translocate nutrients from the soil to the root cortex where transfer to the plant occurs. There is often a dramatic response to mycorrhiza in such soils while, in contrast, there is little effect on the uptake of readily mobile ions such as nitrate. Experiments with ³²P-labelled phosphate indicate that VA mycorrhizal hyphae obtain their extra phosphate from the labile pool rather than by dissolving insoluble phosphates. This may account for plant growth responses reported in silos amended with some phosphate fertiliser. The better utilization of sparingly soluble rock phosphates is explained by the hyphae making closer physical contact than roots with the ions dissociating at the particle surface.

Mycorrhizal benefits are not limited to improved phosphorus uptake. Absorption of other elements including zinc and copper

can be enhanced. Recent work (see Allen *et al.* *Can. J. Bot.* 58, 371; 1980) suggests that growth hormones such as IAA and cytokinins may be present at higher concentrations in plants that are mycorrhizal than in those that are not. There is also some evidence that plants with mycorrhiza may be better equipped to withstand stress conditions such as occur during transplanting or periods of drought, in soils containing toxic levels of aluminium, manganese or salt, or in unstable soils where the hyphae may bring about soil aggregation. In legumes, nodulation and nitrogen fixation have been greatly increased by mycorrhizal inoculation, sometimes beyond that achieved by adding phosphate fertilizer alone. Certain host-fungus combinations may also increase a plant's resistance to some root pathogenic fungi and nematodes. Whether this occurs by direct physical exclusion through competition for sites, or by metabolic changes involving antibiotic production or increased vigour has not yet been clarified. There are situations, however, where the VA fungal drain on host carbohydrate or possible disruption of host metabolism may outweigh the benefits to the plant. This swing from mutualistic symbiosis to parasitism has been shown in some grasses and cereals grown at above optimum phosphate levels.

As the effects of VA mycorrhiza on plant growth are generally positive much research is directed towards harnessing its advantages. This requires an understanding of the physiology and ecology of the association, a knowledge of which crop species or cultivars are most likely to benefit from mycorrhiza, screening and selection of the most efficient fungal endophytes, development of suitable procedures for large-scale inoculum production and inoculation techniques, and an assessment of field situations where mycorrhizal inoculation would be most valuable. Factors that decrease mycorrhiza in the field, for example, pesticides, high fertilizer levels and poor aeration, should also be considered.

Experiments carried out using plants in pots have helped our understanding of mycorrhizal function but they are not always a good guide to responses in the field. Field experiments, however, pose two major problems: how to produce high quality (pathogen-free, high viability) inoculum in quantity (see Menge *et al.*, *Proc. Int. Soc. Citriculture* 1, 129; 1977) and how to inoculate crops on a field scale (see Mosse & Hayman in *Tropical Mycorrhiza Research* ed. Mikola, Oxford University Press, 1980). Inoculum can consist of infested soil (usually from stock plants and containing spores, mycelium and infected root fragments), clean infected roots (as grown in nutrient-flow culture, see Rothamsted Report (1) 188; 1980) or pure fungal cultures (not yet achieved). For

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annual crops sown over large areas inoculum can be pelleted with the seed or placed in the seed furrow either dry or in a slurry. Such material is bulky, yet enough must be applied to ensure substantial early infection when the crop's demand for phosphate, and hence mycorrhiza, is at a peak. Perennial crops are more easily handled since they can be inoculated in containers to produce vigorous mycorrhizal colonization of the root system by appropriate endophytes before transplantation.

Field situations must be selected where mycorrhizal benefits look most probable; crops already known to be responsive to mycorrhiza should be tested in nutrient-stressed soil at sites where the indigenous population of mycorrhizal endophytes is small or inefficient. Current examples are marginal sites in temperate regions (for example, white clover in hill pastures), ferrolateritic soils which are deficient in and/or 'fix' phosphate as found in huge expanses of the tropics (for example, the Brazilian cerrado), eroded, degraded or unstable sites (for example, coal tips,

quarries and sand dunes), and soils fumigated to remove plant pathogens (for example peach and citrus nurseries in California.)

Results so far have shown that growth responses to field inoculation with selected mycorrhizal endophytes can vary from nothing to around threefold. Test crops have included white clover in Britain and New Zealand, lucerne in Spain and Britain, maize and cowpea in Nigeria and cereals in Pakistan. Similar responses have been reported in citrus and up to 84 fold increases in sweetgum in fumigated nurseries in the US. Less dramatic, but nevertheless important responses, are also possible in more standard agricultural soils and techniques to measure these should be refined. In many other situations, however, mycorrhiza may have no discernable effect on crop yield.

At this stage, despite the enormous recent upsurge in mycorrhizal studies, undue faith in mycorrhiza as an agricultural elixir should be tempered by a realistic appraisal of which aspects might best be exploited on a practical scale. □

Tales of two accelerators

from P.I.P. Kalmus

AT 17.00 on 6 June 1978, Nimrod accelerated its last particles. The users, a Queen Mary College — Rutherford Laboratory collaboration, recorded their final events in a kaon-nucleon polarization experiment and ended a fifteen year era during which several hundred scientists from the UK and overseas had used the 7 GeV proton synchrotron at the Rutherford Laboratory. Three weeks later a meeting took place where the events surrounding the conception, construction and life of this machine were recalled by a small number of distinguished speakers.

In September of the following year the 12.5 GeV Zero Gradient Synchrotron of the Argonne National Laboratory in Illinois was closed down, and a two-day symposium was held to recall its history and achievements. The proceedings of both these meetings have recently appeared in print. *Nimrod: The 7 GeV Proton Synchrotron*, edited by John Litt, is available from the Rutherford Laboratory and *History of the ZGS*, edited by Joanne Day, Alan Krisch and Larry Ratner, is published by the American Institute of Physics as No. 60 in its conference proceedings.

It is tempting to contrast the two machines, to compare their performance and to look for different styles of conducting high energy physics in the two

countries, but I will leave that to some future historian of science. Some sociologist might even read national characteristics into the fact that in Britain we waited three weeks before holding the wake whereas our American colleagues held theirs two weeks before the death of the ZGS! However, to me, the similarities between the stories of the two machines and their user communities are much more striking than their differences.

Nimrod was built to enable UK universities to carry out research in the exciting new field of high energy physics. The concept of a national laboratory with central facilities was, however, a novel one in Britain and was viewed with considerable suspicion and apprehension by some prominent university scientists. Similarly, the main purpose of the ZGS was to provide the universities of the American Mid-West with facilities which would rival those on the East and West Coasts, and the idea of basing academic research at a Government laboratory was viewed with hostility by some universities and, in particular, by the Mid-West Universities Research Association.

However, both machines were authorised in the late 1950s. Both made use of the well-established weak focusing principle. Particles make slow oscillations in the horizontal and vertical planes about the idealised orbit. The oscillations have large amplitudes and require a guiding magnet of large aperture. It was felt that the design was safer for achieving high intensity beams than the newly invented

strong focusing principle which was being used in the CERN and Brookhaven machines. These were then under construction and were designed for higher energy but much lower intensity. In fact, the strong focusing machines performed extremely well and their intensities were raised gradually over the years to rival those of the wide aperture machines.

Already during the construction periods the directors of both projects went to considerable efforts to involve members of the university community both in policy making and in the actual design and construction of bubble chambers, beamlines and other facilities. In this both laboratories were highly successful. The fears of university scientists that they would be taken over by Big Brother proved groundless, and in fact Rutherford and Argonne became shining examples of how technologically complex central facilities can serve large user communities. This expertise lives on: the Users Organisation at Fermilab is modelled on that at Argonne and the Rutherford Laboratory (now combined with Appleton) is serving the needs of university scientists and engineers in a variety of fields.

Both accelerators began operation in 1963. Although not the highest energy machines in Europe or America, the range covered by their particles was a particularly fruitful one, the available energy being comparable to the rest mass energy of the stable hadrons and the lower lying unstable particles.

The achievements of the users of Nimrod were primarily in strong interaction physics, in particular, nucleon-nucleon and meson-nucleon interactions. Starting with total and elastic differential cross sections successive generations of experiments measured gradually more complex spin parameters using polarized targets. Much of our present knowledge of hadron spectroscopy stems from this machine. The ZGS, partly because of its higher energy, supported a rather wider variety of experiments including, for example, neutrino interactions in the 12 foot hydrogen bubble chamber. In its final years there was a vigorous programme of physics using an accelerated and extracted beam of polarized protons. The ZGS was the obvious machine for this technique as its uniform magnetic field was likely to cause less beam depolarization than might be the case in other machines.

Well over 200 experiments were conducted at the ZGS and each was a significant research project occupying the effort of perhaps ten physicists for three years. The Nimrod booklet gives a list of 120 PhD theses written on work done with this accelerator.

Both machines were closed prematurely, and both for the same reason — to release funds and support for the new generation of more powerful accelerators at CERN and at Fermilab. The kaon-nucleon experiment that concluded the Nimrod

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programme is now analysed and published. The postgraduate student who worked on it has written his thesis and is embarking on the next stage of his career at CERN. A whole generation of high energy physicists,

some of whom are now at the forefront of particle research, received their training at Nimrod or the ZGS. I personally feel privileged to have participated in a small way in both these adventures. □

Diseases of the motor unit

from a Correspondent

It has been estimated that more than one third of neurological hospital admissions can be attributed to neuromuscular disorders. This large group of diseases was the subject of a recent conference* and, perhaps the major message to emerge was that many basic techniques are now being adapted to serve as specific probes in studying both normal and disordered structure, function and interactions of nerves and muscles. Where such probes have been applied, for example in the case of α -bungarotoxin and myasthenia gravis, rapid progress has been made. The newer techniques offer similar promise for understanding of the muscular dystrophies and other neuromuscular disorders.

The Duchenne form of muscular dystrophy (DMD) is the commonest of the muscular dystrophies and is inherited as a sex-linked recessive trait. It produces progressive muscular weakness in boys and culminates in total disability before the teens. Circumstantial evidence suggests a disorder of the plasma membrane manifested by leakage of muscle-specific enzymes into the circulation, minute breaks in the plasma membrane early in the course of the disease, and a large number of biochemical defects in muscle and other cells. Much evidence was added at the meeting for a membrane or membrane-associated abnormality, although the underlying defect was not defined. Using freeze-fracture techniques Bonilla and Schotland (University of Pennsylvania) found decreased intramembranous particles in sarcolemmal membranes of DMD and facioscapulohumeral dystrophy muscles. These changes suggest defects in the sarcolemma but their significance is not understood. Red blood cells of patients with DMD show increased phosphorylation of a specific membrane protein fraction in band II of spectrin (Roses, Duke University). Although he has identified the sub-fraction responsible for this effect there is little enthusiasm for the idea that the spectrin abnormality could reflect the basic biochemical defect in DMD. Other cell types, such as lymphocytes, may also show membrane abnormalities. Gruemer (Medical College of Virginia) reported that there is decreased capping of DMD lymphocytes upon exposure to anti-human immunoglobulin. Despite the inability of several research groups to confirm these results, Gruemer carried out a double-blind

study in collaboration with the Johns Hopkins group that correctly identified DMD lymphocytes in over 90 percent of samples. In another leukocyte study, Shay (University of Texas) demonstrated a defect of microtubules in mononuclear cells from DMD patients and carriers, using immunofluorescent techniques. A variety of biochemical abnormalities were reported in DMD tissues, including a decrease in adenyl cyclase in sarcolemmal membranes of cultured muscle (Wilner, Columbia University) and an increase in calcium transport by DMD erythrocytes (Pleasure & Mollman, University of Pennsylvania). These disparate findings continue to provide tantalizing evidence for a cell membrane defect in DMD, but are still far from identifying the nature of the defect, or indeed proving that it bears any important relationship to the disease process.

The mechanisms by which abnormal muscle tissue self-destructs may provide more immediate practical applications. There are two proteolytic enzyme systems in muscle fibers (Goldberg, Harvard University), the lysosomal enzymes which degrade normal proteins before their disposal, and an ATP-dependent enzyme system which degrades defective proteins. By blocking the lysosomal enzyme system with leupeptin and other antagonists, muscle breakdown and the degradation of specific proteins can be prevented. Stracher (State University of New York) has applied this interesting approach by using leupeptin in animals with 'dystrophies', or with denervated muscles, and has found a significant retardation of muscle wasting and histological changes.

The changes that occur in muscle cells after breaching the sarcolemmal membrane (as probably occurs in DMD), can be studied by making multiple micropunctures in skeletal muscles *in vivo* (Karpati, Montreal Neurological Institute). The retraction of myofibrils and dilatation of sarcoplasmic reticulum are similar to the changes seen in early lesions of DMD. Perfusion of the limb with EDTA reduces the severity of the lesions, suggesting that calcium may be involved in the destructive process. W. K. Engel (NIH) visualized calcium in human muscle biopsies by a histochemical method. He reported that both damaged and regenerating fibers appear to have elevated calcium concentrations; thus, calcium may contribute to degenerative and regenerative processes in muscle cells. A. Engel (Mayo Clinic) stained muscle biopsies from a wide variety of disease conditions for activated complement

complexes (the 'membrane attack complex'). All muscle fibers undergoing necrosis contained activated complement components regardless of the underlying disease.

These studies all suggest that there are several definable steps between the initial lesion in muscle disorders and the ultimate destruction of the muscle fiber. Therapeutic measures aimed at interfering with these intermediate steps may be able to offer effective treatment.

Developments in the field of myasthenia gravis (MG) have outstripped those in other areas of neuromuscular disease. The availability of α -BuTx (α -bungarotoxin) as a specific probe made possible the identification of the ACh receptor defect nearly 7 years ago. Since that time, much progress has been made in understanding the pathogenesis and devising treatments for this disease. Drachman (Johns Hopkins University) described the mechanisms by which anti-ACh receptor antibodies produce a reduction of available receptors. They include: (1) accelerated endocytosis and degradation of the ACh receptors, (2) blockade of the receptors' active sites, and (3) antibody-mediated, possibly complement-dependent, damage to the receptors and postsynaptic membranes. The mechanism of endocytosis was further elaborated by Pumplin (University of Maryland) and Drachman, who showed by freeze-fracture techniques that cross-linking of ACh receptors by the antibody causes them to clump and aggregate, before their endocytosis *via* coated pits that form in the muscle membrane. Fuchs (Weizmann Institute) reported that immunization of rabbits with denatured ACh receptor can diminish the severity of experimental autoimmune myasthenia gravis (EAMG), presumably by stimulating the production of antibodies that block the binding of more pathogenic antibodies against the native ACh receptor. Another strategy involved the use of anti-idiotypic anti-bodies that appear to protect against EAMG. Such therapeutic approaches may eventually be used in human MG. Lindstrom (Salk Institute) has prepared an extensive collection of monoclonal antibodies against ACh receptor from the electric organs of rays and eels, for the purpose of mapping the structure of the ACh receptors.

Major progress has been made in understanding the 'trophic' interactions between nerves and target cells. ACh transmission may account for the motor nerve's role in regulating the resting membrane potential (RMP) and the extrajunctional ACh receptors of skeletal muscle (Drachman, Pestronk & Stanley, Johns Hopkins University). Post-synaptic blockade of ACh transmission completely reproduces the effects of surgical denervation on the RMP and the extrajunctional ACh receptors, while disuse results in a partial denervation-like effect on these two muscle parameters. The formation of junctions

*A conference "Diseases of the Motor Unit" was held at Key Biscayne, Florida, sponsored by the Muscular Dystrophy Association, June 2-6, 1980.

between nerves and muscles induces the accumulation of ACh receptors (Fischbach, Harvard University) and a low molecular weight substance from chick spinal cord and brain that produces an increase in ACh receptors of cultured muscle has been extracted. The implication of this and other similar studies is that such substances may play a role in the subsynaptic accumulation of ACh receptors at neuromuscular junctions.

Reverse trophic influences of the innervated target cells on neurons within the chick ciliary ganglion were described by Landmesser (Yale University) & Pilar, (University of Connecticut). During development, an excessive number of nerve cells compete to innervate a limited number of target cells; those neurons that form connections are able to survive and mature, while those that fail to connect undergo cell death. The proportion of cells that die can be greatly increased by ablating the target and conversely, the proportion of survivors can be increased by pruning out some of the ciliary neurons. The same effect is also seen in tissue culture; the maturation and survival of ciliary neurons is enhanced by co-culture with an appropriate target tissue, skeletal muscle.

The regulation of nerve sprouting was analyzed by Pestronk and Drachman. Pharmacological denervation (using blockers of nerve conduction of ACh release) induces sprouting of nerve terminals which branch and enlarge over the surface of each muscle fiber. Extrajunctional ACh receptors appear to play a role in this type of sprouting, since blockade by α -BuTx prevents the formation of terminal sprouts. However, in collateral sprouting occurring when neighboring muscle fibers are denervated little effect is produced by blockade of ACh receptors. Finally, intrinsic factors such as age may greatly effect the nerve's ability to sprout — both terminal sprouting and regeneration after injury are progressively impaired with increasing age.

The basement membrane of the motor endplate may be particularly important in neuromuscular junction formation (McMahan, Harvard University) Even after destruction of skeletal muscle cells, the basement membrane retains its organization, and is capable of serving as a target for regenerating motor nerves. Moreover, the basement membrane appears to act as a scaffold for the reorganization of regenerating muscle. If a muscle is damaged and then allowed to regenerate, new endplates form at their previous sites, as directed by the remaining basement membrane.

Extracellular matrix may also play a role in interactions between Schwann cells and axons. The 'dystrophic' mouse shows a defect in myelination of nerve roots, with some Schwann cells apparently refusing to ensheath the naked axons. Aguayo (Montreal General Hospital) showed that grafting segments of unmyelinated nerve

roots from 'dystrophic' mice to normal or even 'dystrophic' mice resulted in correction of the myelination defect. Bunge (Washington University) was able to reproduce the myelination defect in tissue culture of 'dystrophic' mouse dorsal root ganglia, and to correct the abnormality by the addition of fibroblasts or of serum. This suggests that the lack of an extracellular substance normally secreted by Schwann cells results in impaired myelination.

Perhaps the greatest promise for future research in neuromuscular disease lies in the application of a variety of powerful new biological techniques to the eminently workable nerve-muscle system. Among the new techniques described at the conference were monoclonal antibodies to serve as probes of surface membrane components

of chick skeletal muscle (Fambrough, Carnegie Institute of Washington) and tissue culture of pathological muscle. Successful culture of skeletal muscle from patients with myotonic dystrophy was demonstrated by Appel (Baylor College of Medicine) and abnormalities in cultured Schwann cells from patients with adrenal leukodystrophy and Issac's syndrome by Askanas (NIH). Finally, the techniques of modern biochemical genetics are just beginning to be applied to skeletal muscle. Recombinant techniques have been used to clone genes for contractile proteins by Yaffe (Weizmann Institute) and Schwartz (Baylor College of Medicine). H. Epstein (Baylor College of Medicine) pointed out the encouraging prospects for identifying the genetic abnormalities in the muscular dystrophies by such methods. □

Crystallizing membrane proteins

from Richard Henderson

After many years of disappointing failures the first successful attempts to crystallize membrane proteins have recently been reported. Large three-dimensional crystals suitable for X-ray analysis are needed for the resolution of the protein structure in atomic and chemical detail, a level of analysis essential for the understanding of phenomena such as channel selectivity and the mechanism of action of membrane bound pumps and receptors.

In the first paper, Ozawa *et al.* (*Proc. natn. Acad. Sci. U.S.A.* 77, 928; 1980) describe crystals of the cytochrome c oxidase-cytochrome c complex. These crystals were made from detergent (cholate)-solubilised oxidase almost completely depleted of lipid (< 1%). Cytochrome c was added to make a 1:1 complex and crystals obtained by removal of detergent by dialysis. The crystals contained both oxidase and cytochrome c in a 1:1 ratio, but both detergent and lipid were absent. They are thus well-characterised chemically but the extent of crystalline order remains to be determined by X-ray diffraction. At the moment, they are needles rather too small for analysis.

The most successful and promising reports used the detergent, octylglucoside, recently made commercially available. In two exciting reports, crystals were made by precipitation of membrane proteins solubilised in octyl glucoside without removal of the detergent. The crystals thus contained a substantial amount of detergent in addition to aqueous regions, the protein and perhaps also some lipid.

Michel and Oesterhelt (*Proc. natn. Acad. Sci. U.S.A.* 77, 1283; 1980) used 2.5

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— 2.8 M salt to precipitate octyl glucoside-solubilised bacteriorhodopsin, obtaining two crystal forms, one needle-shaped and the other cube-shaped. These crystals are truly three-dimensional and one form (the needle) is ordered to at least 8 Å resolution. The needles have the interesting property of being strongly dichroic and the purple chromophore is oriented almost exactly along the needle axis. Further structural work should therefore be useful to determine the chromophore orientation and if better diffraction patterns can be obtained, the crystals may well be suitable for higher resolution analysis.

Garavito and Rosenbusch (*J. Cell Biol.* 86, 327; 1980), used polyethylene glycol to precipitate the pore-forming protein, porin that comes from *E. coli*. outer membranes, after solubilising with octyl glucoside. They obtained birefringent rhombic plates and prisms using β -octyl glucoside and non-birefringent but larger, bipyramids using α -octyl glucoside which they had to synthesize themselves. The large bipyramids gave X-ray diffraction spots out to 3.8 Å resolution making them ordered at least to a resolution close to that required to reveal atomic detail.

This recent flurry of successful crystallizations may now mean that membrane proteins are sufficiently purified and characterised, and that detergent development is sufficiently well-advanced, that a substantial effort to crystallize many more membrane proteins should be started. Perhaps these results, exciting in themselves, will provide the impetus for these efforts. It seems that it would now be worthwhile both to try to crystallize other membrane proteins and to develop other detergents like octyl-glucoside, which itself remains in solution while the protein-detergent complex is precipitated. □

REVIEW ARTICLE

Transcription and RNA processing by the DNA tumour viruses

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Messenger RNA synthesis by the DNA tumour viruses proceeds by a complex but versatile series of transcription and RNA processing steps. The major mechanistic features of this pathway are probably very similar to those used by the animal cell host itself. The viruses have, however, evolved intricate arrangements of protein coding sequences and sites for RNA initiation, polyadenylation and splicing which allow them to use their genetic information to maximum advantage.

THE DNA viruses which infect animal cells and transcribe their messenger RNA in the host nucleus provide an attractive and easily manipulated system for studying eukaryotic gene expression. The phenomenon of messenger RNA splicing, now recognized as occurring throughout the eukaryotic kingdom, was first discovered in the human adenovirus. This virus, together with the simian virus 40 (SV40) and the mouse virus, polyoma, which will also be considered in this article, realises the full potential of RNA splicing to assemble a range of different messages from a limited amount of primary genetic information. To maximize the efficiency of their relatively small genomes, they have evolved complex transcription units in which the primary transcripts can be processed in different ways to yield different end products (for a review of transcription unit design see ref. 1). Adenovirus-2 (Ad-2, and the very similar Ad-5) is a member of the group C human adenoviruses, and transcribes its RNA from a 35,000-nucleotide linear DNA genome. SV40 and polyoma are both papovaviruses, with covalently closed circular supercoiled genomes ~5,200 nucleotides long, and closely related genetic organization and mRNA maps. (The closely related human papovavirus BK² will not be discussed here.) This review summarizes the arrangement of genes and messenger RNAs within the three viruses, and compares mRNA synthesis pathways to illustrate common mechanistic features. In addition to productive infections, all these viruses can cause neoplastic transformation in certain cell types, but this will not be considered here.

Transcription of adenovirus-2

Ad-2 productive infection of HeLa cells proceeds through a ~36-h infectious cycle which is conventionally divided into early and late stages, separated by the onset of viral DNA replication approximately 8 h after infection (for a review see ref. 3). Ad-2 transcription is by the host RNA polymerase II^{4,5} and both DNA strands encode mRNA^{6,7}.

Transcription during the early stage follows a complex programme. There are at least five early transcription units (shown in Fig. 1) to which mRNA species and proteins have been mapped⁸⁻¹⁶. Each of these, including EIA and EIB, the two tandem transcription units at the left end, has a separate promoter, demonstrated by nascent chain analysis¹⁷, UV transcription mapping^{18,19} or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) inhibition of transcription²⁰. Structurally distinct capped mRNA 5' termini²¹ are encoded by each of the early regions and have been mapped within sequenced DNA templates²². Although unique caps were found for region EIA and EIB²², the caps for EII²³, EIII, EIV and the protein IX

mRNA are heterogeneous, and result from staggered 5' termini encoded within a 2-6-base region (C. C. Baker and E.B.Z., unpublished results). The staggering is non-random and favours the use of purines rather than neighbouring (or intervening) pyrimidines, reminiscent of the prokaryotic initiation preference for purines (for a review, see ref. 24). The two left-end transcription units EIA and EIB encode functions capable of cell transformation²⁵⁻²⁸, and have been partially sequenced in Ad-5 (refs 29, 30). Region EIA products are also implicated in the efficient production of mRNAs from the other early regions (see below)³¹. Mutants in region EIB are complemented by human embryonic kidney cell functions, and this region may encode the Ad-2-specific tumour antigen³². Region EII encodes a 72,000 molecular weight DNA binding protein (DBP) which participates in DNA replication^{33,34} and may regulate early transcription during the late stage (see below). Region EIII is distinguished by its location wholly within the major late transcription unit and encodes a glycoprotein which appears on the cell surface^{35,36}. EIII and EIV yield mRNAs with complex splicing patterns^{12,14}; notably, two 3' termini have been detected for mRNAs with the region EIII 5' end. Two additional leftward regions which are tentatively allocated to the early class are known. One encodes the IVA2 protein, a minor component of the virion; the second, which maps between map coordinates 19.8 and 23.5, encodes low abundance mRNAs and is the locus of the ts36 DNA minus mutation³⁷.

Modulation of early mRNA synthesis

Ad-2 early transcription units are not activated simultaneously, EIA being expressed within 1 h of infection, EIB, EIII and EIV somewhat later, and region EII within 2-3 h of infection³⁸.

The expression of transcription units at the left end of the genome is of particular interest. These encode multiple mRNA species which have common 5' and 3' termini but differ in the internal arrangement of their splices^{8,12,14-16}. During the early stage of infection, region EIA encodes two 13S species, EIA-a and EIA-b shown in Fig. 1B. The donor splice points for these species differ by approximately 200 residues and their DNA sequences have been mapped³⁹. The alternative splices change the internal peptides of region EIA proteins without altering the amino or carboxy termini. These EIA transcripts are very rapidly transported to the cytoplasm, and have a short half life⁴⁰. Region EIB encodes two species, EIB-a, a 22S mRNA, and EIB-b, a 13S species^{12,14,41}. During the late stage of infection, the proportions of the different left-end mRNAs change to favour the splicing pattern of the shorter species. A new 9S mRNA, EIA-c, appears from EIA, and the amount of EIB-b

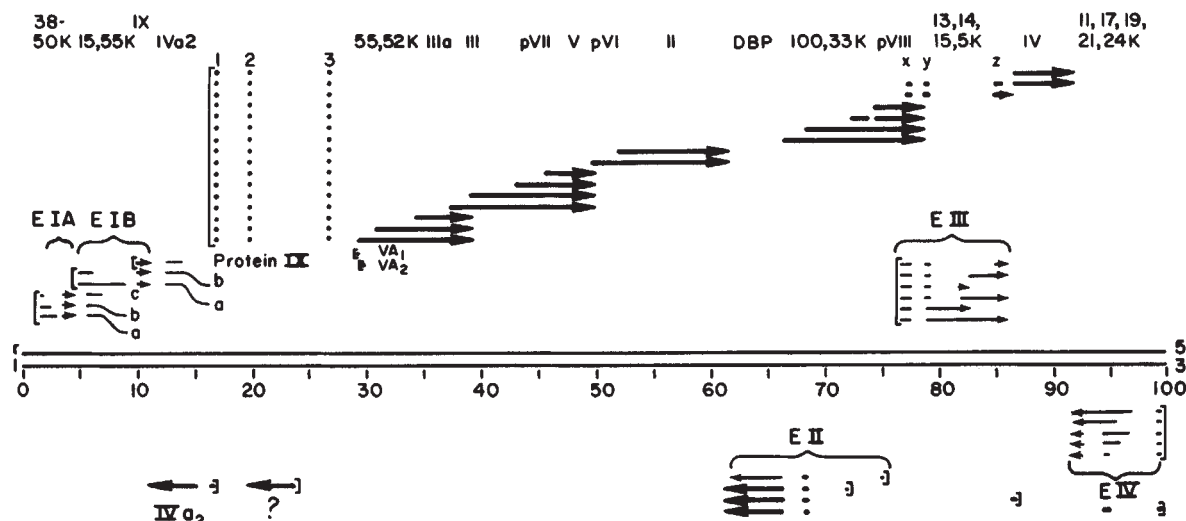


Fig. 1 Synthesis of messenger RNA by adenovirus-2. The physical map of Ad-2 is divided into 100 units, each consisting of ~350 nucleotides. RNAs synthesized from left to right utilize the DNA r-strand as template and are 'rightward' transcripts. Those transcribed in the opposite direction are 'leftward'. A, Transcription and processing of late messenger RNA. Precursors to late messenger RNA are rightward molecules initiated at the major late promoter and are capped while nascent RNA. Five sites, ① to ⑤, exist for the polyadenylation of these transcripts. However, only one poly(A) site is used for any given transcript, although on average the five sites are used with approximately equal frequency. RNA polymerase transcribes beyond the functional poly(A) site, ③ in this example, and continues until it reaches the far end of the genome at coordinate 100. Soon after the polymerase passes ③, while the RNA is still a nascent molecule, the transcript is cleaved to expose the poly(A) acceptor. RNA sequences encoded at coordinates 16, 19 and 27 are spliced together to form a non-coding tripartite 5' leader. This leader is subsequently spliced adjacent to a coding sequence. Several alternatives are possible for this final splicing step when any particular poly(A) site has been utilized, for example joining the leader to *a* or to *b*. In the example shown, splicing to *a* juxtaposes the leader to the coding sequences for the pVI protein, while splicing to *b* splices the leader adjacent to the hexon coding sequence. The mRNA produced by splicing to *a* contains the coding sequences for both pVI and hexon. However, eukaryotic ribosomes do not re-initiate translation at internal positions, and translation of this multicistronic mRNA is confined to the first cistron, pVI. Hexon is translated from mRNA with leader spliced to *b*. Two 'choices', selection of the poly (A) site and the leader splicing site, therefore govern the mRNA species which is produced from the primary transcript. This processing pathway allows 14 or more different proteins to be encoded within a single transcription unit, and places their expression under the control of one promoter. B, Adenovirus-2 messenger RNA maps. Early messenger RNAs fall within five separate transcription units, E1A-E1V. Each early transcription unit encodes multiple species which differ in the positions of their internal splices. The mRNAs encoding the proteins IX and IVa2 are expressed at an intermediate stage of infection. Species from the major late transcription unit fall into five 3'-co-terminal families with all members of a given family sharing a common poly(A) site. The mRNAs from this transcription unit also share a common 5'-capped tripartite leader. For mRNAs within a 3'-co-terminal family, this leader is spliced adjacent to different coding sequences. Other leader sequences, *x*, *y* and *z*, appear in some fibre (protein IV) mRNA molecules. The protein translation products of various mRNAs are aligned with their genes. The VA RNAs are RNA polymerase III products described in the text. See text for refs. B is redrawn from ref. 16.

accumulating in the cytoplasm increases dramatically by 50–100-fold^{12,40,41}.

Further evidence for the temporal complexity of transcription comes from the Ad-2 protein IX^{42,43}. Its mRNA is synthesized later than the early mRNAs but synthesis still occurs in the presence of cycloheximide or cytosine arabinoside (Ara C), which block late mRNA⁴⁴. The protein IX mRNA is therefore assigned to an 'intermediate' stage of transcription. It is unspliced and has a 3' terminus that coincides with that of the EIB mRNAs, but its 5' end is encoded at coordinate 9.7, about 1,800 residues downstream from the EIB cap site^{42,43}. Nascent transcripts initiated at 9.7 are not found in the early state nor is the protein IX mRNA detected in the cytoplasm at this time. Both are detected during the late stage¹⁹. These experiments strongly indicate that these are separate promoters for the protein IX mRNA and the EIB mRNAs, and that the 5' end of the protein IX mRNA is not formed by cleavage of a larger EIB transcript, thus suggesting transcriptional control.

Ad-2 early proteins may participate in the control of Ad-2 early gene expression. Region EIA mutants yield diminished quantities of early mRNAs from the EIB and EII-IV transcription units^{31,45,46}, suggesting that a product encoded by EIA may be required for efficient expression of the other early regions. It is noteworthy that transcription from each early region occurs in the presence of a variety of protein synthesis inhibitors^{38,47}, suggesting that synthesis of an EIA protein is not an absolute requirement for expression of the other early regions.

Early transcription units are also subject to negative regulation. When early RNA synthesis is monitored in the presence of Ara C, a drug which blocks both DNA synthesis and most late mRNA, early viral transcription is shut down approximately 6 h post-infection^{48–50}. However, if protein synthesis is blocked by cycloheximide, early mRNA is overproduced (or stabilized) relative to the wild type, suggesting that negative control of early transcription (or mRNA stability) depends on protein synthesis⁵¹.

The pertinent viral regulatory protein blocked by cycloheximide may be the DBP, the translation product of early region EII⁵². The DBP has a single-stranded DNA binding function in viral DNA replication, but also has the capacity to bind to DNA termini or double strands⁵³. Several facts suggest a complex role for the DBP in adenovirus gene expression. With ts125, a mutant of Ad-5 that produces a temperature-sensitive DBP, early transcription does not undergo its normal shut-off^{48–50}. A detailed analysis of early transcription with cells infected with ts125 shows that the major negative effect of the protein is on region EIV, and that control of the rate of synthesis of region EII is normal in ts125 infection at the non-permissive temperature⁵⁴. The apparent negative regulatory action of the DBP on early region EIV could parallel the proposed SV40 large T antigen autoregulation (see below), which is thought to act at the RNA initiation step. In this connection, it is of interest that the DBP and SV40 T antigen are related by their capacity to change Ad-2 host range. Although Ad-2 expresses both early functions and certain late functions, including replication of its DNA in monkey cells^{55–57}, a greatly decreased yield of infectious particles is obtained⁵⁸. The cause of the block imposed by monkey cells is unknown, but certain late proteins are synthesized in decreased yield⁵⁹, possibly as a result of reduced levels of synthesis of functional mRNAs^{60,61}. Simultaneous infection with SV40 overcomes the host range block. Expression of the sequences coding for the proline-rich carboxy-terminal segment of the SV40 T antigen (see below) is implicated as the relevant SV40 function because Ad-SV40 hybrid recombinant viruses which encode this segment of T are also non-defective in monkey cells^{62–64}. Also, SV40 mutants with deletions at the 3' end of the early region that remove C-terminal peptides (Fig. 3B) are defective in adenovirus helper function⁶⁵. Microinjection of a related Ad-2-SV40 hybrid protein which contains the C-terminal peptide also overcomes the block⁶⁶. Mutants of Ad-2 which overcome the block have been selected, and marker

rescue experiments have mapped the locus of the mutation within the DBP gene⁶⁷. The mutant DBP thus seems to fulfill a function required during monkey cell infection that can also be provided by SV40 T antigen.

The full story of Ad-2 early regulation is likely to be even more complex. During the early stage of infection, the 5' end of region EII mRNA is encoded near coordinate 75 (ref. 23). However, during the late stage a considerable fraction of the mRNA from region EII has a 5' leader encoded at either coordinates 72 or at 86 (refs 12, 16). Thus, there is an activation of 'late specific' 5' ends for transcripts of 'early' region EII DNA. In agreement, synthesis of this early protein continues into the late phase⁶⁸.

The early to late switch

The major shift in Ad-2 gene expression is the early to late switch, which is closely coupled to the onset of DNA replication and dramatically increases the abundance of late transcription unit mRNAs. These messengers contain a common tripartite 5' leader (whose detection marked the discovery of splicing) which is encoded at coordinates 16, 19 and 27 on the genome as shown in Fig. 1B^{69–74}. The 5' terminus of this leader is released by T1 RNase as an undecanucleotide⁷⁵ which is encoded at coordinate 16.45 (ref. 73). With the onset of the late stage, transport of cell mRNA to the cytoplasm ceases, although synthesis of polyadenylated cellular nuclear RNA continues⁶⁸.

Although the mechanism of this shift is not known, it has been established that blockage of DNA replication by chemical inhibitors of DNA synthesis such as Ara C¹² or by DNA minus conditional lethal mutations^{76,77} prevents the formation of most late mRNA. Thus, ts125 fails to synthesize late mRNA if placed at the non-permissive temperature during the early stage of infection⁷⁷. It also fails to replicate its DNA³³. As found for ts mutants of SV40 T antigen at the non-permissive temperature⁷⁸, the onset of DNA replication (rather than the maintenance of synthesis) seems to be the critical factor for late mRNA synthesis. If ts125 is shifted to the non-permissive temperature during the late phase, late RNA synthesis continues although DNA replication ceases⁷⁷. Late mRNA synthesis is also dependent (at least indirectly) on protein synthesis, and late mRNA (as well as DNA replication) is blocked if cycloheximide is added early in infection. The block to late mRNA caused by these drugs may not be absolute because the coordinate 16.4 'late' capped 5' terminus is found in cycloheximide-treated cells early in infection (E.B.Z. and A. Shaw, unpublished results). In the presence of Ara C, late mRNA species with their 3' end at coordinate 38 are found¹².

Virus promoters and primary transcripts

The precursors to mRNAs from the major late transcription unit are large rightward-transcribed nuclear molecules up to 25 kilobases long^{79–81}. The initiation site for late RNA (5' terminus of the primary transcript) was mapped at coordinates 16.3–16.5 by the nascent chain method applied to isolated nuclei⁸² and *in vivo*⁷³. The RNA initiation site was mapped more precisely by showing that late nuclear RNA contains marker oligonucleotides encoded just downstream from the late cap site but lacks sequences predicted by the DNA structure immediately upstream⁷³. This was confirmed for transcripts synthesized *in vivo* and in isolated nuclei^{22,82,83}. Coincidence of the promoter and the cap site implies that the initiating residues of the primary transcript are incorporated into the capped 5' terminus of late mRNA.

RNA initiation site structures

Many eukaryotic structural genes contain a region of homology⁸⁴ which precedes the mRNA cap site by 30 or 31 residues and has the canonical structure TATAAA (the Hogness box). The presence of the Hogness box in the Ad-2 late promoter⁷³ and at a constant location with respect to cell cap sites, suggests that it is involved in transcription initiation. Other regions of homology are shared between the late promoter and

the globin^{73,85} and conalbumin genes⁸⁶. The majority of the Ad-2 early cap site sequences also share a Hogness box²², suggesting that each early cap site is also an RNA polymerase II initiation site. Region EII seems to lack a Hogness box²³, as do the late SV40 and polyoma cap sites (see below), but the significance of this difference is not known.

Regulation of the late primary transcript

Initiation of a capped transcript at the late promoter has been obtained *in vitro* with a soluble system using Ad-2 late promoter DNA, RNA polymerase II from HeLa cells as well as polymerase from various heterologous sources including *Xenopus*, and an S-100 fraction from uninfected cells⁸⁷. 'Fingerprint' analysis of these *in vitro* transcripts gave the pattern of RNase T1 oligonucleotides found *in vivo*, indicating selective and accurate initiation at the cap site *in vitro*. Extracts of whole uninfected HeLa cells also initiate capped late promoter transcripts up to 5,000 nucleotides long, without polymerase supplementation⁸⁸. Moreover, the latter system also initiates at the cap sites for the early promoters EIA and EIB, and the intermediate promoter protein IX.

The fact that for correct initiation *in vitro*, the Ad-2 late promoter (as well as early and intermediate ones) requires no viral component other than the cloned 5' segment of the transcription unit suggests that no virus-encoded factor is required to activate this promoter, at least when it is present as naked DNA. Late transcription could be negatively controlled at the initiation step by a 'repressor', or the late promoter could be active at all times, with the primary transcript changing from non-productive to productive transcripts. Indeed, late transcripts proximal to the coordinate 16.5 promoter are hypermolar in comparison with downstream (coordinates 30–100) sequences⁸². Thus, a proportion of late transcripts are terminated before they reach the major blocks of late genes. A similar (although not necessarily related) observation has been made for RNA synthesized in the presence of DRB^{20,89}. This drug acts at a site downstream from the promoter, and limits transcripts to promoter-proximal (potentially the attenuated) sequences⁹⁰.

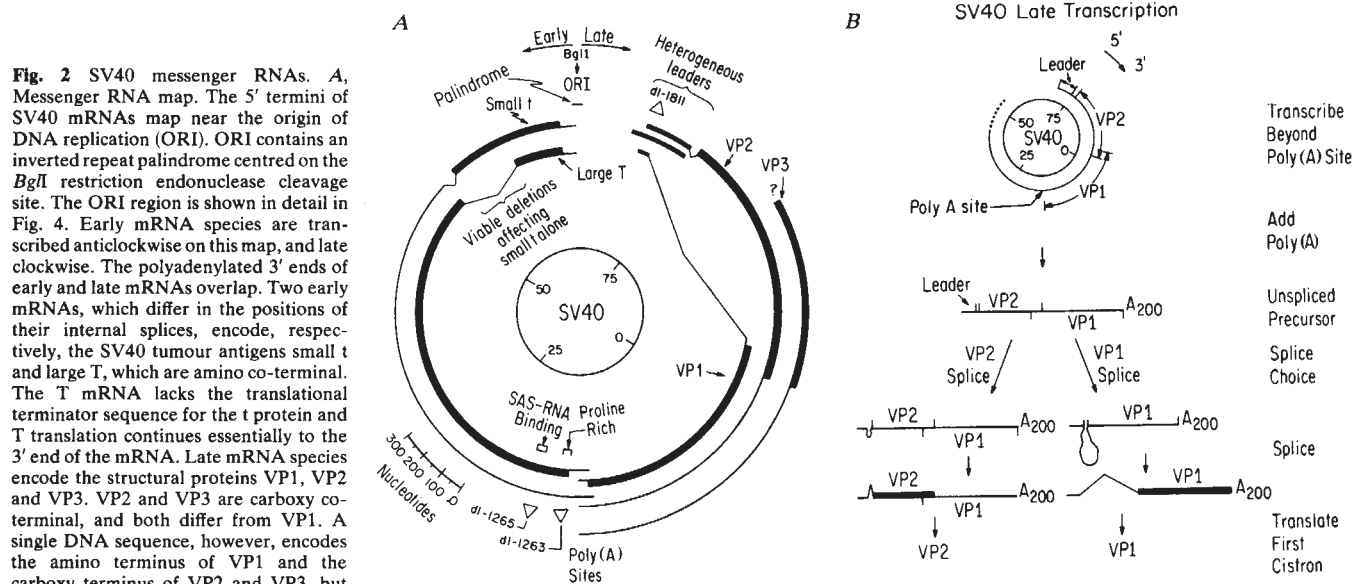
Processing of late transcripts

The pathway for processing late mRNA is given in Fig. 1A. The 3' ends of mRNAs from the late transcription unit are encoded

at five poly(A) sites, each to the right of coordinate 16 (ref. 91). These have been mapped precisely at coordinates 38, 50, 62, 78 and 92 (refs 16, 92–94). Radiolabelling studies indicate that RNA sequences in polyadenylated nuclear molecules adjacent to each of these five poly(A) sites are labelled with equal kinetics in time periods less than required to complete the transcript, implying that nascent RNA is the poly(A) acceptor⁹⁵. The size of the newly polyadenylated RNAs implies that they retain essentially all their intervening sequences, and received their poly(A) before splicing occurred⁹⁵. Unspliced polyadenylated molecules are also observed by electron microscopy⁹⁶ and RNase T1 oligonucleotides from the late Ad-2 first intervening sequence are found in nuclear molecules with 3' poly(A)⁷³. However, with such large RNAs it is difficult to exclude the possibility of the excision of short stretches before poly(A) addition occurs. Isolated nuclei also synthesize polyadenylated transcripts with 3' ends at coordinates 38, 50, 62 and 78, coincident with the *in vivo* poly(A) sites⁹⁷. The nonpolyadenylated fraction of this RNA, however, also includes species with 3' termini at coordinates 38 and 50, as well as a proportion of large molecules with partially spliced 5' leaders. Although some splices may occur on non-polyadenylated RNA, the final splice of the leader to the mRNA main body seems to be confined exclusively to the polyadenylated RNA class.

With early regions EII and EIV, polyadenylated nuclear RNAs are also readily detected with the size and map position expected for unspliced but capped precursors^{98–100}. Studies on the production of mRNA from early regions EII and EIV have determined the size of the molecule to which labelled poly(A) becomes attached after brief exposure to ³H-adenosine. Over 90% of the new poly(A) was on unspliced molecules¹⁰¹. If the unspliced, polyadenylated precursor to region EII mRNA is labelled *in vivo*, and nuclei are isolated and incubated with cell extracts, this RNA is spliced *in vitro*¹⁰². The general rule seems to be that poly(A) addition precedes splicing, but it is not known whether this effect is a consequence of rapid poly(A) addition and slow splicing, or whether poly(A) addition is a prerequisite for splicing.

Late in infection, Ad-2 transcription occurs in equimolar amounts along the entire late transcription unit (with the exception of hypermolar transcription near the promoter discussed above)^{80,95}. The primary transcripts initiated at coordinate 16 extend beyond the five poly(A) sites, and proceed virtually to the



end of the genome at coordinate 100 (ref. 103). At this stage of infection, mRNA 3' ends are apparently generated by cleavage. Products from both the 5' and 3' sides of the cleavage have been detected in isolated nuclei⁹⁷. Although only approximately one-fifth of the RNA mass reaches the cytoplasm^{95,104}, polyadenylated sequences are transported almost quantitatively⁹⁵. This implies that there are no non-productive polyadenylation events, and that only one poly(A) tract is added per late transcript although all five potential acceptors are transcribed. Thus, polymerases can evidently traverse one poly(A) site non-productively, utilize a second downstream site and avoid non-productive polyadenylation at the remaining positions, although they are transcribed and the acceptor is a nascent RNA. This suggests that the transcription complex actively prevents non-productive poly(A) addition, possibly through a regulated commitment of the polyadenylation machinery to one of the poly(A) receptors to the exclusion of the other four, at the time of initiation⁹⁵. Alternatively, the virus may have evolved a gradient of poly(A) site efficiencies which counterbalances map positions and ensures that each poly(A) site is used with approximately equal efficiency. The Ad-2 late 3' acceptors contain the AAUAAA homology found near the poly(A) of cell mRNAs even for termini encoded within the transcription unit, for example, at coordinate 50 (ref. 91).

The number of translatable Ad-2 mRNA species from the late transcription unit¹⁰⁵ is greater than the number of 3' termini. This excess is explained by the existence of messengers which are staggered in sequence at the 5' end (adjacent to the 5' leader), overlap at internal positions, and share common 3'-terminal structures⁹². The late mRNAs are organized into five banks of overlapping messengers (see Fig. 1B)^{91,93,94}. Present evidence suggests that the larger of the 3' co-terminal species (from a given family) contain coding sequences for more than one protein, but that only the 5'-terminal coding sequence is translatable^{92,93,106}. Adjacent sequences, silent in the large mRNAs, are translated from shorter mRNA species belonging to the same 3'-co-terminal message family^{92,93,105-107}. This model is supported by the fact that the 5' leader, sequenced for both the fibre⁷² and the hexon^{108,109} mRNAs, lacks an AUG, but that translation initiation sites are found within the mRNA main body. These late 3'-co-terminal families parallel the papovavirus late 3'-co-terminal mRNAs described below. The late leader also contains a sequence complementary to the 3' end of 18S ribosomal RNA which may facilitate ribosome binding^{73,108}.

If we consider the 3'-co-terminal messengers (and their translation products), the five poly(A) sites divide Ad-2 late protein coding sequences into discrete blocks capable of expressing more than one protein through the formation of 3'-co-terminal mRNAs. With the Ad-2 late transcription unit, two post-transcriptional 'choices' (outlined in Fig. 1A) presumably govern which segment of nuclear RNA will be transported to the cytoplasm as mRNA: selection of the poly(A) site from one of five positions within the late transcription unit followed by selection of the site to which the leader is spliced. All these events are nuclear¹¹⁰. The actual mechanism for removing the intervening sequences may be quite complex. Late nuclear RNA contains species which have the leader assembled as far as 19.3, 21.6, 24.8, 25.6, 26.0, 26.2, 26.4 and 26.9 coordinate map units and which are colinear downstream⁹⁶. These results suggest that the primary pathway for processing late nuclear RNAs has a sequential polarity for splicing from 5' to 3'. In some cases incompletely spliced mRNAs are transported to the cytoplasm. With the fibre mRNA, and a related mRNA synthesized by an Ad-2-SV40 hybrid virus, a proportion of the molecules contain up to three extra leader segments, *x*, *y* and *z* of Fig. 1B; however, these do not affect translation^{111,112}. With infections in monkey cells, a proportion of the fibre mRNAs retain even larger abnormal RNA sequences which are derived from coordinates 66-78 and are normally present only in mRNAs from the fourth co-terminal block⁶¹. Thus, the monkey cell block (see above) may result from defective splicing or premature trans-

port. Sequences used for both early and late splicing show extensive homology with equivalent cell sequences, implying common sequence recognition for the cell and at least some splices of both early and late viral transcripts^{23,73,113}.

Although the mechanism of splicing is not established, most models postulate an intramolecular mechanism (see Fig. 1A), perhaps with small RNAs bridging the splice junctions and holding the exon sequences in close proximity to allow accurate cleavage and religation¹¹⁴. With Ad-2, attractive candidates for this role are the RNA polymerase III-encoded virus-associated (VA) RNAs. Two prominent species, 5.5S and 5.2S (about 156 nucleotides long), are encoded by genes which lie just in front of the late gene block, close to coordinates 29-30 on the Ad-2 map (Fig. 1B) and separated by a short spacer¹¹⁵⁻¹¹⁸. Both species of VA RNA appear during early infection, but the 5.2S species levels off and the 5.5S species increases during the late stage¹¹⁶. Splicing models have been proposed in which VA RNA base-pairs with splice junctions of late transcripts¹¹⁹.

In addition to polyadenylation and splicing, late mRNAs are modified by methylation of internal A residues at the *N*⁶ position¹²⁰. These are added in the nucleus before splicing and (in contrast to the bulk of the RNA transcripts) are highly conserved on transport to the cytoplasm, suggesting that they may play a part in designating which sequences from the large transcript are to appear as mRNA.

mRNA arrangement for SV40 and polyoma

As with adenovirus, productive infections of SV40 and polyoma involve the temporal regulation of gene expression, with the synthesis of 'early' and 'late' mRNAs at different stages in infection. The early and late mRNAs of SV40 and polyoma are transcribed with opposite polarity with respect to the DNA physical map, and use opposing DNA strands as templates. Their 5' termini are, however, clustered within a region of the genome that includes the origin of DNA replication¹²¹⁻¹²⁴. Because the complete DNA sequences of SV40^{125,126} and polyoma¹²⁷⁻¹³² have been determined, precise gene and mRNA mapping is possible.

The arrangement of SV40 genes and transcripts is shown in Fig. 2. During the first 8-10 h of SV40 infection, mRNAs are synthesized for two early proteins, the 90-100 kilodalton large T antigen¹³³ required for viral DNA replication, and the 15 kilodalton small t antigen¹³⁴⁻¹³⁷ implicated in cell transformation (for a review see ref. 38). The mRNAs for SV40 T and t have closely mapping or identical 5' and 3' termini, but differ in their splicing^{139,140}. The RNA encoding t is colinear with the DNA sequence from an AUG at coordinate 66 to a terminator at 55 (refs 140-142), but is followed by a downstream splice which falls completely within the untranslated region. In the T mRNA, the 5' side of this splice shifts to remove the terminator and allow T translation to continue essentially to the 3' end at coordinate 17 (ref. 140). The T and t proteins thus share amino-terminal sequences, but differ at their carboxy termini, and the smaller protein is encoded by the larger mRNA¹⁴³⁻¹⁴⁵. As a consequence of the early splicing patterns, deletions in DNA exclusive to t shown in Fig. 2A do not affect T synthesis¹⁴⁶. Recently, a SV40-associated small RNA (SAS RNA) of unknown function, induced late in infection, has been identified that can associate with early viral mRNA near the 3' end of the coding region (Fig. 3A) (ref. 147 and P. Berg, personal communication). If small RNAs indeed mediate splicing, a third splicing arrangement for SV40 'early' messengers may exist.

The messenger RNA map of polyoma is shown in Fig. 3A. Initiation of early transcription probably occurs near the beginning of the early region, because a defective genome that contains only tandemly repeated copies of the replication origin is an efficient template for early RNA synthesis¹⁴⁸. With polyoma, evidence for organization of early genes is provided by alignment of open reading frames in early DNA with known early proteins encoded by the wild type and by deletion mutants. This analysis identifies early coding regions with splices analogous to those for SV40 T and t (refs 138, 149-151) and predicts

proteins with highly homologous amino acid sequences^{127,132,152}. As with SV40, deletions in the *t* gene between coordinates 80 and 85 (hr-*t* deletions shown in Fig. 3A) do not change the apparent size of T¹⁵³. Downstream from the *t* gene, two reading frames are open for translation, suggesting that the *T* gene is overlapped by a second, out of phase, coding sequence. Polyoma encodes a third major early protein, the 55 kilodalton middle *T*, which shares amino-terminal peptides with *T* and *t* but differs at the carboxy terminus¹⁵⁴⁻¹⁵⁹. Messenger RNA for *T* is synthesized according to a third early splicing pattern which also removes the *t* terminator, but joins the coding sequences for the amino terminus to the second large open reading frame, which is shifted with respect to the *T* frame. The frame-shifted overlap of polyoma middle *T* and *T* mRNAs results in differing carboxy termini for the two antigens, encoded by the same DNA segment, and is reminiscent of the cistron *D-E* and *A-B* overlaps in Φ X174 (ref. 158).

Both polyoma and SV40 encode three late proteins, VP1, VP2 and VP3, which are structural components of the virion^{131,159}. Mapping of nascent late transcripts places the start site for the late SV40 transcription a short distance upstream from coordinate 0.735, very near the region of the DNA which encodes the late mRNA 5' termini, as shown in Fig. 2B, and suggests transcription initiation at the cap sites¹⁶⁰. The SV40 late messengers have heterogeneous capped 5' termini encoded in a ~400-residue region adjacent to the replication origin (Fig. 4)¹⁶¹⁻¹⁶³. As with Ad-2, capped purine 5' ends predominate greatly over pyrimidines. Two 5' termini starting with AUU account for the majority of the species^{164,165}. A deletion mutant of SV40, d1-1811, mapped in Fig. 3B, which lacks the major cap site is, however, viable and transcribes mRNAs which have 5' termini that are minor components in a wild-type infection¹⁶⁶. Polyoma late mRNAs also have heterogeneous 5' termini. Three of these have been mapped in the genomes by sequencing and arise from a cluster, again with purines favoured over pyrimidines¹⁶⁷.

T antigen function

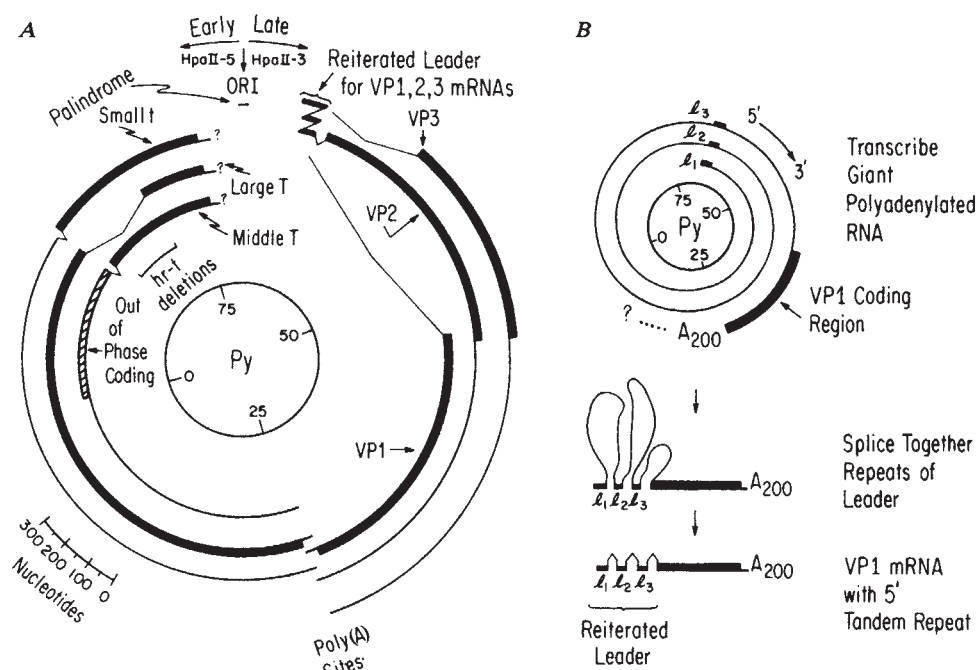
SV40 *T* antigen, as well as a protein encoded by the adenovirus-SV40 hybrid D2 which contains the carboxy-terminal sequence

of *T* antigen, binds to the SV40 DNA replication origin, protecting the region shown in Fig. 4^{168,169}. The 5' end of the early (*T* antigen) messenger RNA is encoded within the binding site for this antigen¹⁴⁰. Thus, *T* antigen may regulate its own synthesis, possibly through binding to the initiation site for its mRNA. This idea is supported by the overproduction of early mRNA by ts *A* mutants of SV40 at the non-permissive temperature¹⁶⁹⁻¹⁷⁴. Post-transcriptional steps may also regulate early mRNA synthesis. In undifferentiated embryonal carcinoma cells, early mRNA is transcribed but it is neither spliced nor transported to the cytoplasm¹⁷⁶. However, when these cells are stimulated by retinoic acid to differentiate, early transcripts are processed into early mRNA¹⁷⁵.

The replication origin

In binding to the SV40 origin, successive *T* antigen molecules are added in the direction of the late cap site(s)¹⁶⁹. Functional *T* antigen is required for the initiation of each round of DNA replication^{177,178}, and DNA replication is in turn required for the onset of the late stage of transcription. However, if replication has already commenced, a temperature shift of a ts *T* antigen mutant which halts DNA replication permits continued transcription of late RNA¹⁷⁹. Late RNA sequences are also detected in the nucleus at low levels at early times before DNA replication¹⁸⁰⁻¹⁸², or when DNA replication has been blocked with ts *T* antigen mutants¹⁸³. Similarly, polyoma 'late' sequences may be detected at early times, before DNA replication, at a stage when late mRNA is not yet being synthesized¹⁸⁴. These observations imply that late RNA synthesis is not strictly dependent upon DNA replication. SV40 DNA may be isolated as a 'minichromosome' complex which contains histones in a beaded nucleosomal structure¹⁸⁵. A short region of this complex which extends from the origin of DNA replication to the late cap sites (coordinates 0.67 to 0.73, Fig. 4) is preferentially cleaved by a variety of nucleases, suggesting that these residues are exposed to the solvent, rather than being masked by the histones of nucleosomal beads¹⁸⁶⁻¹⁸⁸. This same region contains the proposed SV40 RNA initiation sites, and if this structure is maintained *in vivo*, exposure of the putative promoters to the

Fig. 3 Polyoma messenger RNAs. **A**, Messenger RNA map. As with SV40 (Fig. 2), the 5' termini of polyoma mRNAs map near the origin of DNA replication (ORI). The sequence of the ORI contains an inverted repeat palindrome which overlaps the junction of fragments 3 and 5 from *Hpa*II restriction endonuclease digests of polyoma DNA. Early mRNAs are from anticlockwise transcripts, and late from clockwise transcripts. Polyoma synthesizes three early mRNA species which encode the tumour antigens small *t*, large *T*, and middle *T*. Although the exact splice points for the early mRNAs have not been determined, the *t* and *T* messengers have splicing patterns comparable to those of the equivalent SV40 mRNAs. With the mRNA for *t*, splicing allows translation to continue beyond the *t* gene. Translation enters the region encoding *T*, however, in a second out of phase reading frame generating a novel carboxy-terminal sequence for *T*. The arrangement of coding sequences in the polyoma late messengers is analogous to those of SV40 (see Fig. 2) and the polyoma mRNAs also have heterogeneous capped 5' termini (not shown). The 5' leader, however, contains a novel tandemly reiterated sequence (see below). The hr-*t* deletions are discussed in the text. **B**, Polyoma VP1 mRNA synthesis. At the late stage of infection, polyoma polyadenylated nuclear RNAs are large molecules encoded by polymerases which make several transits of the circular genome. These polymerases can transcribe the late poly(A) acceptor sequence without addition of poly(A), on one transit, but use the poly(A) acceptor sequence on a subsequent pass (compare with late Ad-2 in Fig. 1). It is not established whether this 3' end is formed by RNA cleavage. The mature mRNA contains a 5' leader with tandem repeats (*l*₁, *l*₂, *l*₃) of a sequence that exists only once in the genome. The reiteration results from the splicing together of each occurrence of this sequence within the giant polyadenylated RNA and joining the repeated structure to the VP1 (shown here), VP2 or VP3 coding sequences.



See text for refs.

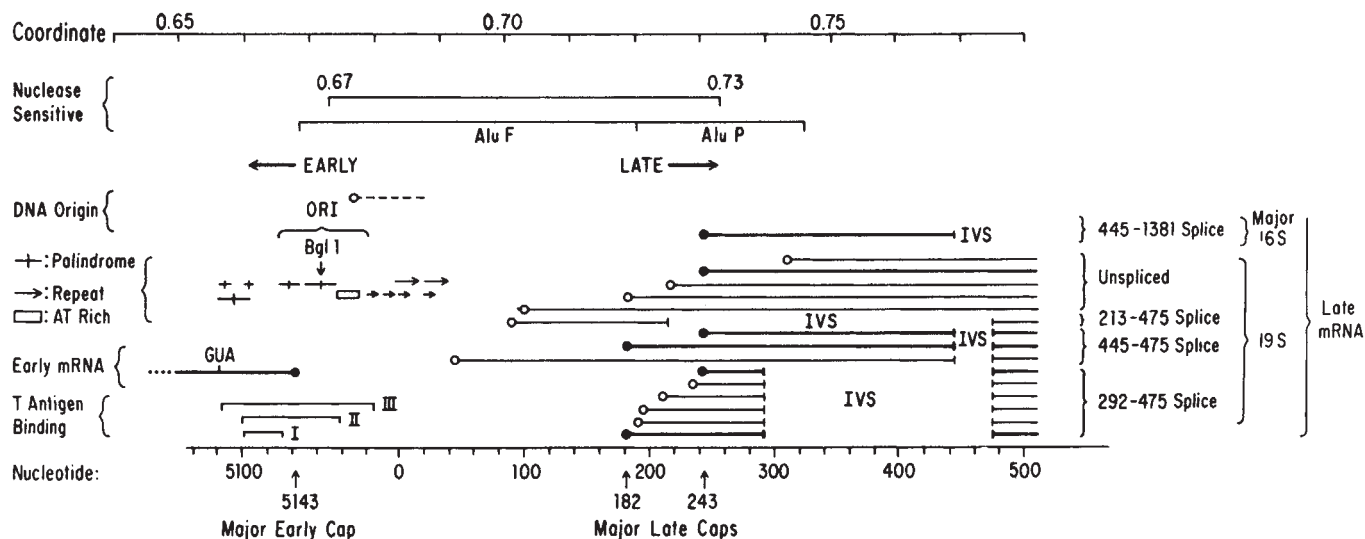


Fig. 4 Organization of transcripts and DNA sequences within the ORI region of SV40. A detailed map of the DNA replication origin (ORI) region of SV40 between coordinates 0.64 and 0.78 is shown (compare with Fig. 2). This region encodes the cap structure (●, major species; ○, minor species) of SV40 early (transcribed 5' to 3' leftward here) and late (transcribed right) mRNAs. The AUG of early mRNA which encodes the amino terminus of T and t tumour antigens is shown. The limits of ORI defined by deletion mutants are indicated. ORI contains a 27-base inverted repeat sequence centred on the *Bgl*I restriction endonuclease cleavage site, and is flanked by various direct and palindromic inverted repeat sequences and an A+T-rich region. The sequence of one arm of the central palindrome is also highly repeated in several animal cell DNAs. The SV40 T tumour antigen, and related proteins, bind as mono-, di- or trimers to the ORI DNA and protect, respectively, sequences I-III from DNase digestion. Because the cap site for early mRNA lies within this binding site, T antigen may autogenously regulate its synthesis by repressing transcription of its mRNA. The capped 5' termini of late mRNAs are heterogeneous and encoded within a ~400-nucleotide region adjacent to the ORI. SV40 minichromosomes are sensitive to nuclease in this region. DNase 1 preferentially cleaves between coordinates 0.67 and 0.75 and restriction enzymes such as *Alu*I release the restriction fragments *Alu* F and *Alu* P more rapidly than other fragments. Intervening sequences (IVS) and splice sites for late 19S and 16S mRNAs are shown. See text for refs.

nucleoplasm could facilitate RNA polymerase binding to the DNA.

During bi-directional SV40 DNA replication, if new histone synthesis is blocked by cycloheximide, parental histones distribute themselves asymmetrically, with 80–90% segregating with the 'leading' side of the two DNA replication forks¹⁸⁹. With SV40, the parental DNA which is the template for the leading side of DNA replication (at both forks) is also the template for mRNA. In the cell, nucleosomes also preferentially associate with DNA template strands which code for stable cell nuclear RNA. Thus, replication origins and RNA initiation sites may have similar arrangements in the cell and the virus¹⁸⁹.

It has recently been shown that a major class of DNA repeat sequences found in animal cells contains a 15-nucleotide sequence which is homologous with a sequence at the SV40 DNA replication origin¹⁹⁰. The 15-nucleotide sequence forms one half of a palindrome which is centred about the *Bgl*I cut site of the virus at coordinate 67 (Fig. 4). In the cell, these sequences are interspersed between many genes¹⁹¹, including those of the mouse β -globin gene cluster¹⁹⁰ where, in an *in vitro* transcription system, they are transcribed by RNA polymerase III¹⁹². The homologous sequence is also found at the DNA replication origins of polyoma, BK virus and hepatitis B virus, and within a small RNA which hydrogen bonds to mRNA¹⁹⁰. Although it is possible that these repeats in the cell are DNA replication origins interspersed between transcription units, and are cellular targets of T antigen, their function has not been established.

Formation of the polyadenylated 3' end

The polyadenylated 3' ends of SV40 early and late mRNA converge at coordinate 17 and overlap¹²⁶. As indicated in Fig. 3B, SV40 late transcription continues in an equimolar fashion at least 1,000 residues beyond the poly(A) site of late mRNA, implying that a cleavage event forms the 3' end^{160,193}. With polyoma, polyadenylated late nuclear transcripts are giant RNA molecules (see Fig. 3B), two or three times larger than genome length^{194,195}. These probably result from multiple transits of the polymerase around the circular genome. Just as polymerases transcribing Ad-2 late RNA can pass five poly(A) sites but use only one, so polymerases may make several passes across the late polyoma poly(A) site before a functional poly(A) receptor is

synthesized. For both papovaviruses, the AAUAAA hexanucleotide homology is found adjacent to early and late poly(A) sites^{126,131}.

Splicing papovavirus late mRNA

The predominant late RNA sequences in the nucleus are unspliced and extend from the cap site(s) to the poly(A) site. This suggests that capping and polyadenylation precede splicing¹⁹³. Splicing of SV40 yields, with approximately equal efficiency¹⁹⁶, two major size classes of mRNA: 18S–19S, which encodes VP2 and VP3, and 16S, which encodes VP1^{126,197,198}. At least two alternative forms of 19S mRNA are found which differ in the splicing of the 5' leader (Fig. 4)¹⁶². For both forms, the 5' leader is non-coding, and the same AUG which lies within the main body of the mRNA is utilized for initiation of VP2 translation¹⁶². To form the VP1 mRNA, a much larger intervening sequence is removed, and the leader is juxtaposed to a second downstream AUG which initiates VP1 translation¹⁹⁹. In fact, this AUG lies within the VP2 gene, but the VP1 and VP2 gene sequences which overlap are read in different frames. The mRNA for VP3 is not well characterized, but the gene for this third late SV40 protein forms a 3'-co-terminal overlap with the VP2 mRNA, and utilizes an internal AUG codon of VP2 for the start of translation²⁰⁰. The choices of splice available for forming SV40 late 3'-co-terminal mRNA shown in Fig. 2B directly parallels the Ad-2 splice choice of Fig. 1.

Late mRNA species from SV40 can survive the deletion of large segments of their leaders without loss of function^{201,210,211}. Integrity of the splicing sites themselves, however, is a prerequisite for stable mRNA synthesis. If the splice junction on one or both sides of an intervening sequence is deleted, the transcript is destabilized and is degraded in the nucleus^{202–205}. The short sequence homologies at splice sites, GU at the donor (5') side and AG at the acceptor (3') side, found in cellular genes²⁰⁶, are also found for SV40 (ref. 162).

Although the arrangement of late genes and mRNAs in polyoma^{207,208} is very similar to that in SV40, the existence of the giant polyadenylated polyoma nuclear species apparently permits splicing mechanisms to form mature mRNA leaders with a novel structure. The polyoma late mRNAs contain a sequence which is reiterated in the 5' leader, but which occurs

only once in the DNA²⁰⁹. In the processing model which explains this paradox (Fig. 3B), giant polyoma transcripts are spliced to join together each occurrence of the repeat sequence in giant RNA to form a reiterated leader structure²⁰⁹. SV40 late mRNA also has reiterated leaders, but with a lower frequency, consistent with the shorter average size of nuclear transcripts²¹².

Conclusions

Studies of DNA virus transcription suggest a common pathway for the synthesis of many viral mRNAs that includes initiation at the cap site, transcription of the mRNA precursor beyond the functional poly(A) site, cleavage and polyadenylation to form

the 3' end, followed by splicing and transport. Many features of this pathway are likely to be shared with the cell. The viruses have, however, evolved complex arrangements of coding sequences and sites for initiation, splicing and polyadenylation of RNA which use the pathway to maximum advantage. The next task is to identify the steps in these pathways that are regulated, and to identify the viral and cellular components which exert control.

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ARTICLES

Rapid polymer transport in concentrated solutions through the formation of ordered structures

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Our previous finding that high molecular weight polymers in certain systems can be transported with an unexpectedly high apparent 'diffusion' rate in the presence of a concentrated second polymer has been shown to be due to the formation and movement of organized macroscopic structures in an otherwise convection-free solution. The structures are not formed as part of a normal phase separation but will dissipate with time. Their formation may be understood by recently developed principles of nonlinear nonequilibrium thermodynamics.

OUR studies on the mechanism of macromolecular transport in gels and polymeric networks in solution have been primarily concerned with relating our observations to biological transport and organizational phenomena, in particular to transport phenomena occurring in the extracellular matrices of connective tissues and within passive membranous structures (for reviews see refs 1, 2). We now present results concerning the kinetics and ultimate formation of ordered structures arising from concentration gradients in aqueous multicomponent polymer solutions.

Initial observations of rapid transport of macromolecules in polymer networks

Preston and Snowden³ observed that polyethylene glycol and polyvinyl alcohol exhibited higher migration rates in polysaccharide solutions than in polysaccharide-free media. Similar

experiments with compact globular solutes exhibited reduced transport rates which were explained by hindered diffusion^{4–6}. A detailed re-investigation⁷ of the ternary polymer–polymer–solvent systems, using a capillary technique⁸, confirmed the earlier studies. Some of the results of this investigation have been reported^{9,10}. The transport of a linear flexible macromolecule (component 1) in solutions of a polysaccharide (component 2) was found to show a marked dependence on (1) time, (2) molecular weight (MW) and concentration gradient of component 1, and (3) MW and concentration of component 2.

As an example of several systems studied we consider the migration of polyvinylpyrrolidone (PVP) (MW $\sim 3.6 \times 10^5$; $D = 2.1 \times 10^{-7}$ cm² s⁻¹) as component 1 in a solution of dextran (MW $\sim 10^4$) as component 2. As described previously^{7,9,10}, at low concentrations of component 2 (C_2), a slight reduction in the transport rate of component 1 was observed. As C_2 was increased, the migration of component 1 increased to a rate 40 times higher than that observed in the absence of component 2,

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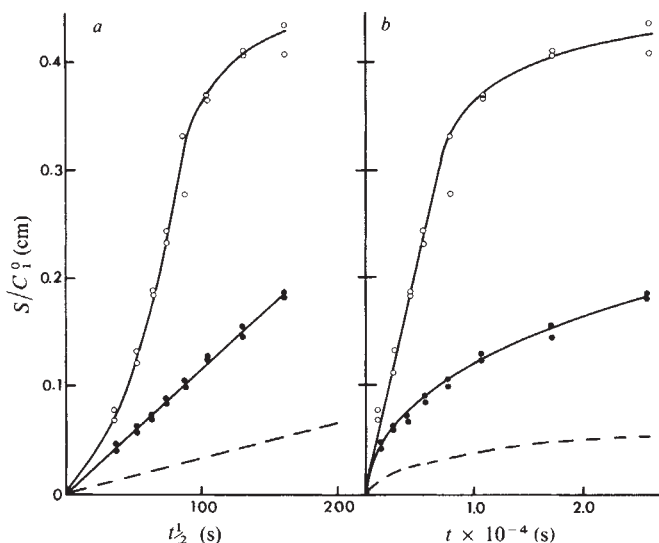


Fig. 1 Transport of ^3H -PVP ($\circ$) and ^{14}C -sorbitol ($\bullet$) over a boundary formed by layering 135 mg ml^{-1} dextran ($\text{MW} \sim 10^4$) over 5 mg ml^{-1} ^3H -PVP ($\text{MW} \sim 3.6 \times 10^5$), 10 mg ml^{-1} ^{14}C -sorbitol and 135 mg ml^{-1} dextran. S is the amount transported per cm^2 of surface area and C_1^0 the initial concentration in the lower compartment. For normal diffusion the transport should be proportional to $(t)^{1/2}$, as shown for sorbitol but not for PVP (a). Instead, the PVP transport is directly proportional to time before it levels off (b). For comparison, the normal diffusion of ^3H -PVP in water is included (---). Experiments were carried out at 20°C .

and then, decreased at still higher values of C_2 . When the transport of glucose or sorbitol ($D = 58 \times 10^{-7}\text{ cm}^2\text{ s}^{-1}$) as a solvent marker was measured simultaneously with the migration of component 1, the behaviour of the low molecular weight solute was normal in that its transport rate was reduced ($D = 39 \times 10^{-7}\text{ cm}^2\text{ s}^{-1}$) by the presence of component 2. This suggests that gravitational instabilities¹¹ had not led to macroscopic convective destruction of the boundary. As ternary polymer-polymer-solvent systems are known to be affected by phase transitions¹², it was possible that the enhanced migration rates were linked to the kinetics of phase transitions. We therefore established⁷ that the systems under study were in regions far from phase transitions.

The time dependence of the apparent diffusion rate of component 1 was very marked in the initial stages. The rates reported¹⁰ were those measured after an interval of 41 h, where they exhibited only a slight time dependence. Our qualitative interpretation of these effects was focused on the hydrodynamic and thermodynamic parameters considered important in determining diffusion rates in multicomponent systems¹³⁻¹⁵. This approach has been successful in describing polymer diffusion in binary systems¹⁶⁻¹⁸. However, we show here the rapid transport of PVP in dextran solutions is not a normal diffusional process.

Time dependence of PVP transport in dextran solutions

When the transport of PVP in dextran solutions was analysed as a diffusional process, it was found that the migration rate was markedly time dependent. However, as the capillary technique used precluded an accurate analysis of transport rates at early times, we used a new technique to investigate time dependence.

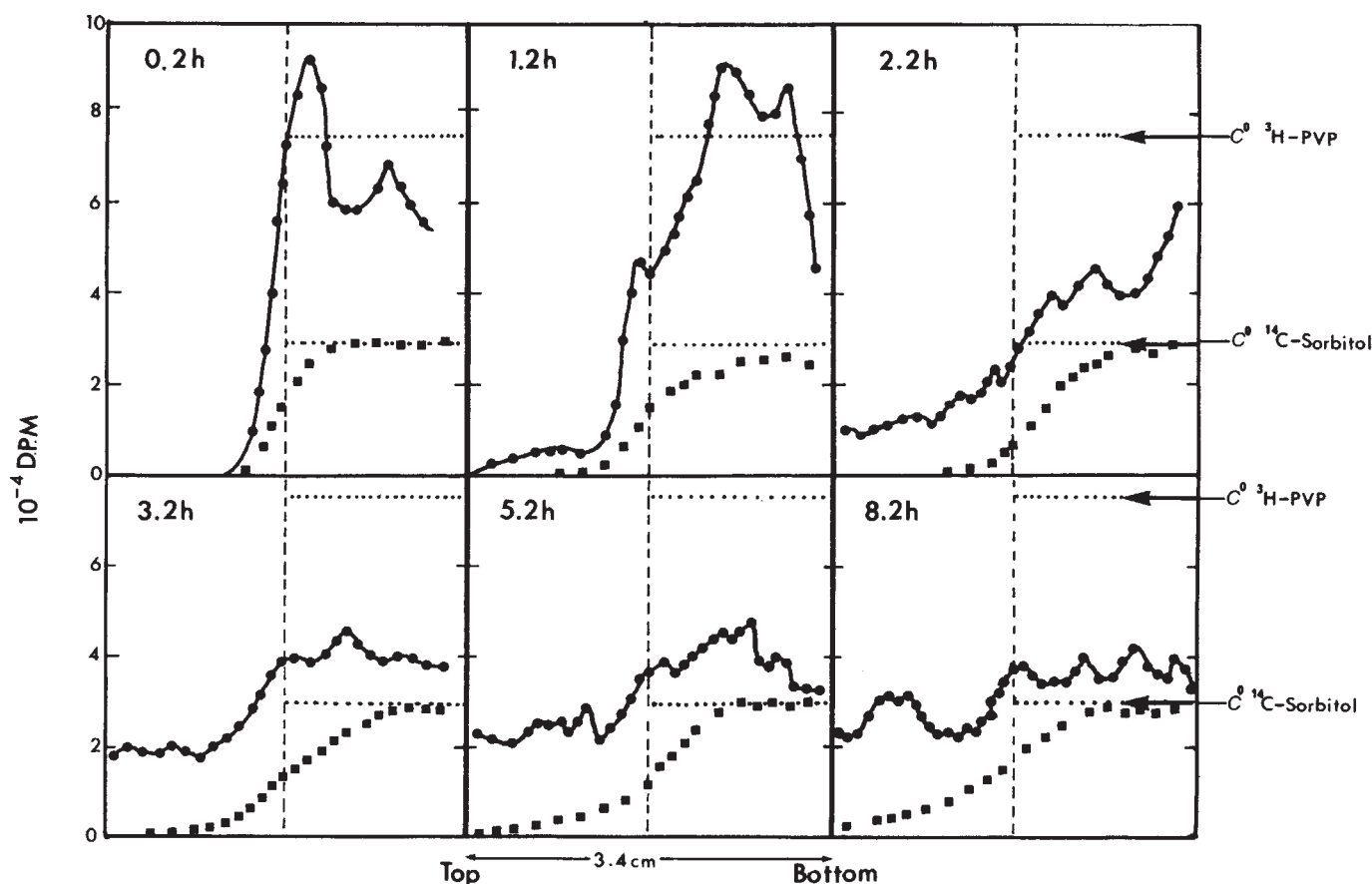


Fig. 2 The solutions described in Fig. 1 were layered in 3.4 cm long plastic tubes. Their contents were fractionated after various times— 0.2 to 8.2 h and the distribution of ^3H -PVP ($\bullet$) and ^{14}C -sorbitol ($\blacksquare$) determined. The change in concentration of ^{14}C -sorbitol is due to normal diffusion, whereas ^3H -PVP spreads more rapidly and exhibits a wave-like distribution. The position of the original boundaries (---) and the original concentrations of the components (...) are indicated. The ordinate represents the radioactivity found in each fraction.

The apparatus consists of a diffusion cell (designed by L. -O. Sundelöf) in which a free horizontal boundary is formed by a shearing mechanism between two solutions of comparable volume. Transport of labelled material across the boundary can be directly monitored as a function of time, by using a number of identical cells. For simplicity we will consider the behaviour of the ^3H -PVP (component 1) in a dextran (component 2) of concentration, $C_2 = 135 \text{ mg ml}^{-1}$. This value of C_2 corresponds to a maximal apparent diffusion rate of component 1¹⁰. Most experiments used a PVP concentration difference of 5 mg ml^{-1} in the presence of a sorbitol concentration difference of 10 mg ml^{-1} , the latter acting as a solvent marker to detect any macroscopic convective flow. Figure 1 shows the time dependence of ^3H -PVP and of the ^{14}C -sorbitol flux in this system. The ordinate represents the ratio of the quantity of solute transferred (S) across the boundary per unit cross-sectional area, to the initial concentration of the solute (C_i) in the lower part of the diffusion cell. When S/C_i is plotted against $(\text{time})^{1/2}$ ($t^{1/2}$) (Fig. 1a), the transport of the ^{14}C -sorbitol follows diffusional kinetics (linear with respect to $(t^{1/2})$) and corresponds to a diffusion coefficient of $\sim 40 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. The transport of the ^3H -PVP follows a sigmoidal pattern, not expected for a normal diffusional process. However, if S/C_i is plotted against time (Fig. 1b), two distinct transport modes are observed for the ^3H -PVP. In the first 3 h, about 40% of the polymer moves across the initial boundary, and the transport rate seems to be linearly related to time. At later times, the rate falls to a much smaller value. The striking differences in the behaviour of PVP and ^{14}C -sorbitol confirms the previous results in that no bulk convective flow is occurring in a system in which high rates of ^3H -PVP transport are observed. Figure 1 includes comparable data describing the transport of ^3H -PVP in the absence of the dextran network.

The linear time dependence of the initial ^3H -PVP transport rate suggests that its migration might be mediated by a convective or gravity-induced process. It is unclear why such movement is not accompanied by a similar behaviour of the solvent marker ^{14}C -sorbitol. However, the diffusion rate of the ^{14}C -sorbitol does increase slightly over that in PVP-free media ($D = 33 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$). Although the transport rates increase with temperature, a correction term allowing for the temperature dependence of the viscosity of water makes the rates temperature independent. This indicates absence of any active process in the transport of ^3H -PVP, suggesting instead that the transport rate is a function of the viscosity of the system. In agreement with this conclusion, we previously reported¹⁰ that the rapid transport is a function of the molecular weight of the network component, we now relate this to the macroscopic viscosity of the system.

To study the spatio-temporal relationships of the process, the experiment was carried out in a vertical centrifuge tube. After layering the dextran solution onto a solution containing dextran, ^3H -PVP and ^{14}C -sorbitol, the tube was allowed to stand for varying periods of time, and was then fractionated by displacement with a dense fluid (tetrabromoethane). The profiles of the distribution of material as a function of time are shown in Fig. 2a-f. Two features of the time evolution of this system are evident: (1) the migration of ^3H -PVP results in a wavelike distribution of material propagating throughout the solution; and (2) ^{14}C -sorbitol shows no comparable behaviour, developing a normal gaussian-like distribution.

Effect of parameters influencing the density differences over the boundary

The direct influence of gravity on PVP transport in dextran solutions was established by monitoring PVP migration in the analytical ultracentrifuge by UV absorption. The boundary conditions (see Fig. 1 legend) were similar to those in experiments performed at unit gravity. We found that the PVP transport rate increased with the centrifugal force acting on the system; however, the relationship was not a simple one. For

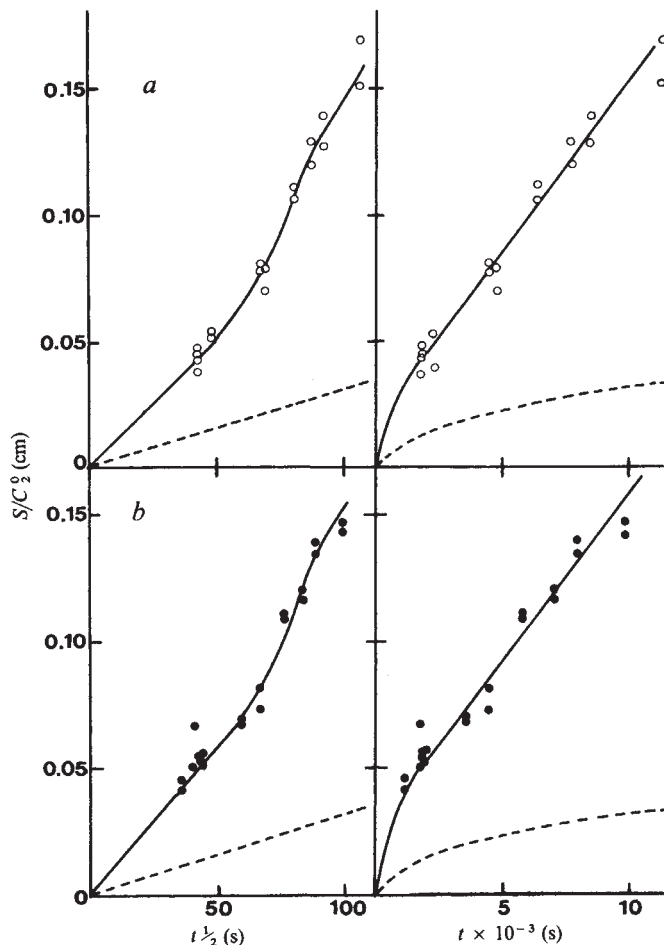


Fig. 3 The upward (a) and downward (b) fluxes of dextran over a boundary similar to that described in Fig. 1 legend except that PVP is unlabelled and dextran labelled with ^3H . For comparison, the normal (trace) diffusion of dextran in a system containing 135 mg ml^{-1} dextran but no PVP has been included (---). The dextran fluxes in the presence of PVP are much higher than in normal diffusion and do not obey diffusion kinetics. Experiments were carried out at 20°C .

example, at unit gravity the rate was 1.4 mm h^{-1} , at $1,077 \text{ g}$ it was 3.8 mm h^{-1} and at $44,000 \text{ g}$ 9.0 mm h^{-1} .

Our attempts to stabilize the boundary with density differences by conventional means did not always produce a 'stabilization' in terms of reducing the enhanced PVP transport. The presence of a sorbitol concentration of 10 mg ml^{-1} in the lower solution or the use of D_2O in the lower solution did not affect the magnitude of the PVP transport. These results suggest that the 'instabilities' caused at the boundary are not affected by the macroscopic density of the solutions as derived from low molecular weight solutes or dense solvents.

Conversely, the PVP transport rate seems to depend critically on the concentration gradients of macromolecular species across the boundary. Variation of the PVP concentration gradient markedly affects the subsequent transport rates⁷. At low PVP concentration gradients no anomalous transport was seen.

Dextran flux in the system

The unidirectional transport rates of ^3H -dextran were measured in both directions across the PVP boundary, and indicated that dextran was also rapidly transported and, as with PVP transport, was essentially linear with time (Fig. 3a, b). The unidirectional rates were of similar magnitude; this means there was only a small resultant downward flux of dextran over the boundary. Figure 3a and b also show the unidirectional transport rates of the dextran in the absence of any PVP gradient; this represents the self (or tracer) diffusion behaviour of dextran at $C_2 = 135 \text{ mg ml}^{-1}$ ($D = 3.6 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$).

Direct visualization of the PVP transport

Direct visualization of the fluxes involved was achieved using a blue-labelled PVP obtained by coupling PVP to remazol brilliant blue (see Fig. 4 legend). When a free boundary was formed in either a thermostatted Tiselius diffusion cell or by manual methods in a test tube or glass cuvette, a striking series of events was seen. After an initial period, a number of uniformly spaced blue vertical 'fingers' or spikes appeared at the boundary (Fig. 4a). These spikes grew at a rate comparable in magnitude to the PVP transport rates given earlier and were associated with a concomitant development and propagation downwards of colourless fingers. In view of our earlier observations, it is tempting to suggest that the polymeric components of the blue advancing fingers are PVP and dextran and that the descending structures contain only dextran. After 2–3 h, the system seemed to reach a 'stable' state in which a regular pattern of alternate blue and colourless lines extended through the whole volume (Fig. 4b). The pattern resembles the salt fingers observed in the double-diffusion system of Turner¹⁹.

To observe the formation of these structured flows from above, the experiments were performed in a Petri dish (32 mm diameter). The dextran solution containing the blue-labelled PVP was layered under a dextran solution of similar concentration but without the PVP. The ensuing series of events was again complex but briefly consisted of the initial formation of needle-like structures of relatively intense blue colour at the perimeter of the dish and directed radially towards the centre (Fig. 5a), followed by the formation of several concentric circles of blue colour parallel to the perimeter of the dish (Fig. 5b). The

two patterns then merged to yield a very complex mosaic-type appearance (Fig. 5c). Within 20 min the regular pattern had begun to disperse and within 2 h a homogeneously coloured solution was obtained.

Variants of the two behaviours described above can be created by using D₂O in the lower solution, which seems to prevent the downward growth of structures, or by changing the boundary conditions (different container shapes). The experiments were also carried out in the dark with similar results, indicating that these events were not light induced, arising from absorption effects.

The apparent generalization of these phenomena or closely related events has been suggested by comparable observations in various ternary polymeric systems under similar constraints.

A tentative explanation of the phenomenon

Our physical picture of the phenomenon supposes concentration inhomogeneities occur in the volume proximal to the initial boundary, so that regions of differing densities are formed. Regions of relatively high density would sink and subsequently displace regions of lower densities; conversely, the low density regions may float and displace regions of higher density. Possible mechanisms leading to density inhomogeneities include a net water flux downwards due to osmotic pressure differences across the boundary, or a net movement of dextran upwards due to the chemical potential gradient induced in the uniform dextran matrix by the presence of the PVP²⁰. This latter effect would increase the concentration of matrix dextran immediately above the initial boundary with consequent sinking

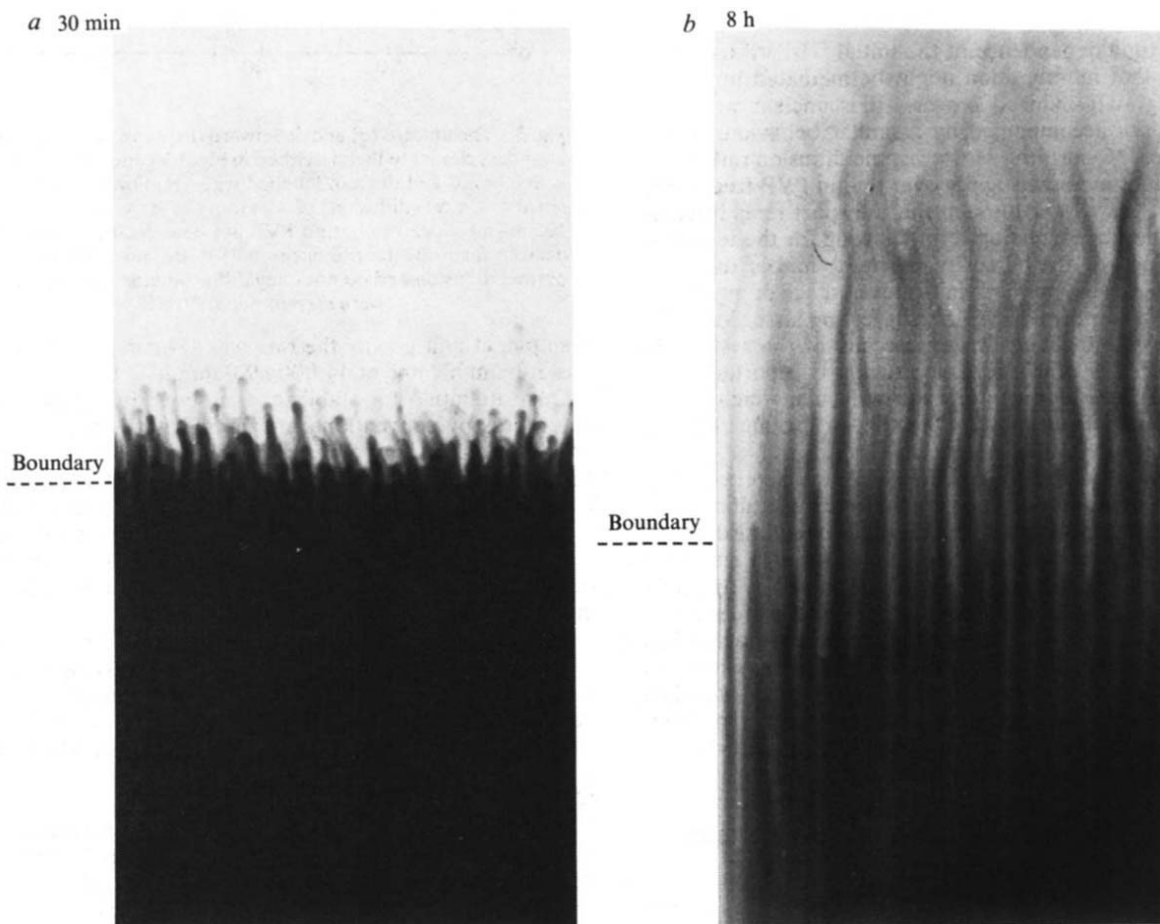


Fig. 4 The formation of structures at the boundary between 135 mg ml⁻¹ dextran (top) and 5 mg ml⁻¹ blue PVP, 135 mg ml⁻¹ dextran and 10 mg ml⁻¹ sorbitol (bottom). Blue PVP was prepared by heating PVP (5 g) and remazol brilliant blue (0.75 g) in NaOH (200 ml, 0.05 M) at 50 °C for 3 days. The coloured polymer was purified by repeated washings with distilled water in Amicon ultrafiltration apparatus equipped with a PM 10 membrane filter, under a pressure of 60 p.s.i. The boundary was formed in the centre of a 6.0 cm long Tiselius cell and photographed 30 min (a) and 8 h (b) after layering. The structures remained in the cell for at least 24 h. $\times 3$.

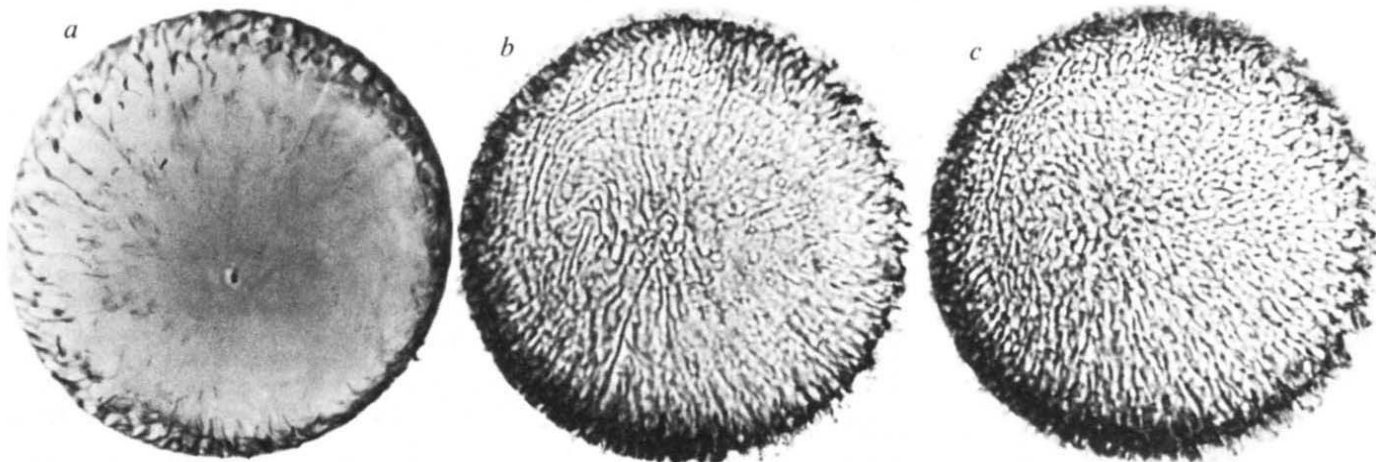


Fig. 5 The formation of structures in a Petri dish seen from above. A boundary between the same solutions described in Fig. 4 legend was formed by layering 1 ml of blue solution under 1 ml of clear dextran solution with a needle. The photographs were taken *a*, 4 min; *b*, 12 min; and *c*, 20 min after the layering. $\times 2$.

of the regions of PVP-free solutions through the layers of solution below the boundary. Alternatively, the intrinsic compositional differences in regions close to the boundary due to thermal fluctuations in polymer concentrations could, without invoking any initial transport across the boundary, create unstable regions which would coalesce and grow—a non-equilibrium nucleation phenomenon of the kind described by Nicolis and Prigogine²¹.

Whatever the precise mechanism of formation, the dense regions may start a downward movement of fingers and displace regions of PVP-containing solution upwards. The slow diffusional characteristics of the PVP will retain this polymer in the upward-going structures and its vertical transport rate will be rapid and basically determined by the bulk movement of the structures. Low molecular weight solutes such as sorbitol will, however, undergo a rapid lateral diffusive exchange between the upward- and downward-moving structures and hence the net transport in the vertical directions will be low and governed by the lateral diffusional process. The observation that high concentrations of low molecular weight solutes (sorbitol or D₂O) in the solution below the boundary do not inhibit the rapid transport of the PVP can be understood in terms of a rapid equilibrium of these solutes between regions close to the boundary where initial polymer concentration inhomogeneities exist. Furthermore, these small molecular weight solutes may equilibrate rapidly between the upward- and downward-moving structures, thereby limiting their 'stabilizing' effect.

Are dissipative structures involved in these systems?

Glansdorff and Prigogine²², through a general formulation of nonequilibrium statistical mechanisms, have concluded that two basic conditions are required for the formation of ordered coherent structures in an open system. First, factors must operate on the system to drive it further from an equilibrium state. Second, nonlinear mechanisms must act between the various elements of the system. When these conditions are met, certain types of fluctuation can be amplified and drive the system to a new regime which differs from the initial reference state. This regime is characterized by the appearance of organizations (called dissipative structures) whose probabilities of occurrence at equilibrium would be negligible. It is important to recognize that nonequilibrium unexpectedly may be the source of order-producing coherent behaviour in space and time.

It seems that the two basic requirements for the formation of self-organizing coherent behaviour are present in our system. First, the PVP concentration gradient will induce a chemical

potential gradient in the dextran network, where no actual dextran concentration gradient exists. Such an induced chemical potential gradient would cause the dextran, initially at uniform concentration, to migrate, thus forcing the system further from the equilibrium state. Second, it is well recognized that the thermodynamic parameter describing the interactions occurring in concentrated polymer systems is nonlinear. We thus tentatively conclude that the observed structures in the ternary systems are an example of dissipative structures.

We believe that our demonstration of self-organization occurring merely by intrinsic heterogeneities in polymer concentration is an important concept for an understanding of the organization and transport of high molecular weight solutes in biological systems. Furthermore, similarities exist between the presently described phenomenon and the 'finger' instabilities arising from the penetration of a fluid into a porous medium containing a more viscous fluid²³. These instabilities are gravity or pressure induced and, when considered in conjunction with our observations, may be particularly relevant to an understanding of the complex transport effects found in the nerve axon.

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Isolation of a yeast centromere and construction of functional small circular chromosomes

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The centromeric DNA (CEN3) from yeast chromosome III has been isolated on a 1.6 kilobase-pair segment of DNA located near the centromere-linked CDC10 locus of Saccharomyces cerevisiae. When present on a plasmid carrying a yeast chromosomal replicator, CEN3 enables that plasmid to function as a chromosome both mitotically and meiotically. Minichromosomes containing CEN3 are stable in mitosis and segregate as ordinary yeast chromosomes in the first and second meiotic divisions.

In an attempt to isolate and characterize yeast centromeric DNA, the techniques of overlap hybridization¹, complementation of mutations in yeast through transformation^{2,3}, immunological screening^{4,5}, and complementation of auxotrophic mutations in *Escherichia coli*⁶ have been used in our laboratory to obtain a number of hybrid plasmids containing DNA segments from around the centromere-linked *leu2*, *cdc10*, and *pgk* loci on chromosome III of *Saccharomyces cerevisiae*. A contiguous region of about 25 kilobase pairs of DNA that extends from the *LEU2* gene to the *CDC10* gene has been obtained on a set of overlapping plasmids^{1,3}. Among these is the plasmid pYe(*CDC10*)1, which contains the yeast *CDC10* gene included on an 8 kilobase-pair segment of yeast DNA³. This plasmid was selected by complementation of the *trp1* and temperature-sensitive *cdc10* lesions in strain XSB52-23C from a pool of hybrid plasmid DNAs composed of segments of sheared yeast DNA joined by poly (dA · dT) connectors into the *E. coli*-yeast shuttle vector, pLC544 (ref. 3). This vector contains the yeast *TRP1* gene along with the chromosomal replicator, *ars1* (ref. 7).

Unlike other *ars1* bearing plasmids described by Stinchcomb *et al.*⁷, Kingsman *et al.*⁸, and below, pYe(*CDC10*)1 is relatively stably maintained through mitotic and meiotic cell divisions in yeast. We have investigated the nature of this stability both biochemically by localization and preliminary sizing of the stabilizing segment of DNA, and genetically by examining the behaviour of pYe(*CDC10*)1 and its derivatives in mitosis, meiosis, sporulation and germination. The stabilizing segment is confined to a 1.6-kilobase pair region on the left (*LEU2*) side of the *CDC10* locus. Its presence on plasmids that also carry a yeast chromosomal replicator [*ars1* (ref. 7) or *ars2* (ref. 9)] permits those plasmids to behave mitotically and meiotically as minichromosomes. Genetic markers on a minichromosome act as linked markers. They segregate in the first meiotic division as centromere-linked genes, are thus present in the two sister progeny of the second meiotic division, and are unlinked to genetic markers on other chromosomes. We conclude that the stabilizing DNA segment from pYe(*CDC10*)1 is a functional centromeric DNA sequence, the centromere of chromosome III (*CEN3*).

Plasmid pYe(*CDC10*)1 is mitotically stable

We have reported previously that in cultures of individual XSB52-23C/pYe(*CDC10*)1 transformants growing at 37 °C in the absence of tryptophan (selective conditions) only about 1–2 cells in 500 examined microscopically have lost the plasmid and express the easily distinguished *Cdc10*[–] phenotype³. Using

overlap hybridization¹, a number of other plasmids have now been identified from our pLC544(*TRP1 ars1*)-yeast DNA genomic library that carry DNA segments from the *cdc10* region of chromosome III (Fig. 1). One of these, pYe98F4T, carries the *CDC10* locus because it complements the *cdc10* mutation in strain XSB52-23C, and it also contains additional DNA in the direction of *PGK*, but lacks about 3.5 kilobase pairs of DNA in the *LEU2* direction that is found on pYe(*CDC10*)1. Microscopic examination of cultures of individual XSB52-23C/pYe98F4T transformants (growing selectively) reveal that approximately one-half of the cells express the mutant *Cdc10*[–] phenotype, indicating extensive plasmid loss and instability. The degree of mitotic stability of pYe(*CDC10*)1, pYe98F4T, and two other *TRP1 ars1* plasmids containing DNA from near the *CDC10* locus, pYe65H3T and pYe101C3T (Fig. 1), was further examined by growing individual cultures of XSB52-23C transformants containing each of these plasmids on rich medium at 23 °C (nonselective conditions). After approximately 20–30 generations of nonselective growth, individual clones of the original transformants were scored for loss of plasmid (loss of the *Trp*⁺ phenotype). As seen in Table 1, 97% of the clones derived from transformants harbouring pYe(*CDC10*)1 retained the plasmid after this extensive period of nonselective growth. Plasmids pYe98F4T, pYe65H3T, and pYe101C3T are completely segregated, however, in the same conditions. The parent vector in the above plasmids, pLC544 (ref. 8), other *TRP1 ars1* plasmids such as YRp7 and YRp7-Sc2604 (ref. 7), plasmids containing the chromosomal replicator (*ars2*; ref. 9), and many plasmids containing 2 µm DNA replicators¹⁰ are also mitotically unstable.

Plasmid pYe(*CDC10*)1 is meiotically stable and segregates as a chromosome

Because stability in mitosis is a predicted property of a replicating unit carrying a functional centromere, the unique mitotic stability of pYe(*CDC10*)1 encouraged us to examine the behaviour of this plasmid through meiosis and sporulation. Strain XSB5223Ca/pYe(*CDC10*)1(*cdc10 leu2 trp1/CDC10 TRP1*) was crossed with X2928-30-1Aa(*trp1 leu1 ade1 met14*), diploids were sporulated and the resulting asci were dissected for genetic analysis. Data from 16 tetrads (cross 1, Table 2) indicate that the plasmid (marked by the wild-type *TRP1* allele) in at least 60% of the asci segregates in the first meiotic division as a chromosome and is thus found in the two sister spores, the products of the second meiotic division. Only parental ditte and nonparental ditte asci were obtained in this cross using as

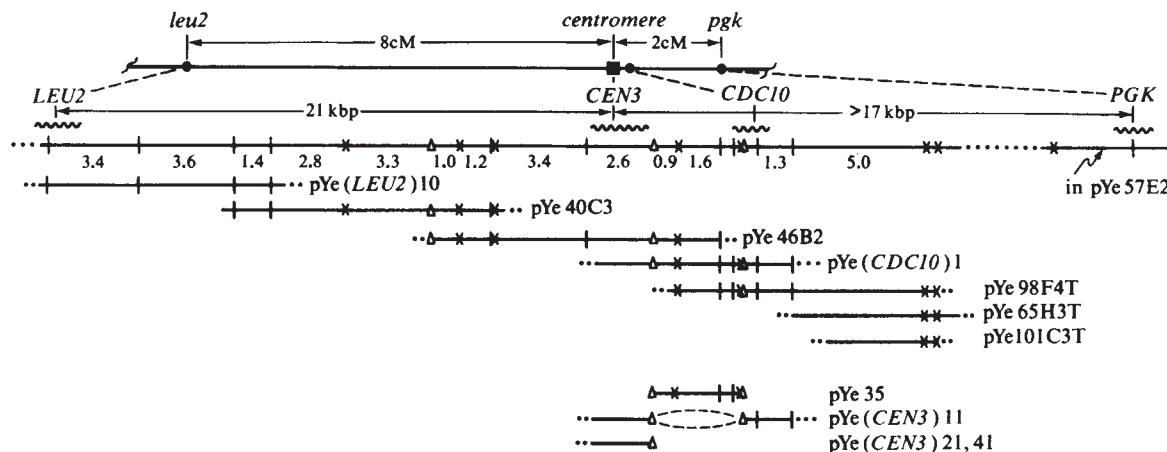


Fig. 1 Genetic and physical maps of the centromere region of *S. cerevisiae* chromosome III. The restriction map gives the location of *Eco*RI (—|—), *Hind*III (—x—), and *Bam*HI (—Δ—) sites in the DNA. Numbers denote kilobase pairs (kbp). The order of inserts in various overlapping plasmids is indicated below the restriction map. Small dots at the ends of inserts pertain to those sheared segments of yeast DNA that are joined to their respective plasmid vectors by poly (dA · dT) connectors. The isolation and characterization of plasmids pYe(*LEU2*)10 (ref. 6), pYe40C3 (ref. 1), pYe46B2 (ref. 1), pYe(*CDC10*)1 (ref. 3), and pYe57E2 (ref. 5), have been described. Plasmids pYe98F4T, pYe65H3T, and pYe101C3T were obtained by overlap hybridization and characterized as described by Chinault and Carbon¹, with the modification that a single restriction fragment (from pYe(*CDC10*)1) purified by agarose gel electrophoresis was used as probe in the hybridizations²⁰. Plasmids pYe(*CDC10*)1, pYe98F4T, pYe65H3T, pYe101C3T, pYe35, and pYe(*CEN3*)11 all contain the *TRP1 ars1* vector, pLC544 (ref. 8). The vector portion of the remaining plasmids is Co1E1.

references the centromere markers *leu1*, *leu2*, *met14*, and *ade1* (complete data not shown). Thus the *TRP1* locus on the plasmid behaves as a centromere-linked marker. The *TRP1* gene on pYe(*CDC10*)1 is unlinked to *ade1*, *cdc10*, *leu1*, *leu2*, or *met14* and is therefore not integrated on chromosomes I, III, VII or XI. The plasmid was completely lost in about 30% of the asci and in one ascus was found in all four spores, but did not segregate either 1+:3- or 3+:1- in any asci analysed in this cross.

A second cross was carried out with one of the progeny of cross 1, SB17Aa/pYe(*CDC10*)1(*cdc10 trp1 leu2 ade1/CDC10 TRP1*), and strain 6204-18Aα (*cdc10 leu2*). Data similar to that from Cross 1 were obtained (cross 2, Table 2). The plasmid in cross 2 again segregates 2+:2- in the first meiotic division in 92% of the asci and is found in all four spores in one ascus. With *ade1* as the reference centromere marker, again the marker scored on the plasmid (*CDC10* in this cross) is centromere-linked. The plasmid marker, *CDC10*, is unlinked to chromosome IV, because in those tetrads where *CDC10* segregated 2+:2-, *TRP1* (wild-type allele on both the minichromosome and one parental chromosome) segregated both 2+:2- and 4+:0-.

Backcrosses of plasmid-bearing progeny from cross 1 with the original parents XSB52-23C and X2928-3D-1A gave the same pattern of 2+:2- segregation of the wild-type plasmid marker that is independent of the segregation of markers on other chromosomes. In all the above crosses, the two markers on pYe(*CDC10*)1, *CDC10* and *TRP1*, were linked in every ascus scored (data not shown). None of the asci obtained consisted of two viable and two nonviable sister spores. Thus the minichromosome does not appear to pair with any of the other yeast chromosomes.

The centromeric DNA (*CEN3*) is located on the left (*LEU2*) side of the *CDC10* locus

The data presented above indicate that plasmid pYe(*CDC10*)1 contains functional centromeric DNA (*CEN3*) from chromosome III. Making use of the set of overlapping plasmids (Fig. 1) and various reclones from the *CDC10* region, we have attempted to determine the location of *CEN3*. In order to locate the *CDC10* gene on pYe(*CDC10*)1 we had previously constructed two reclones of this plasmid³. The first, pYe(*CEN3*)11 (see Fig. 1; referred to as pYe(*CDC10*)1-1 in ref. 3) contains all

the yeast DNA in pYe(*CDC10*)1 cloned into pLC544, except the 3.5-kilobase pair *Bam*HI fragment from the middle of the insert. The second, pYe35 (referred to as pYe(*CDC10*)1-2 in ref. 3) contains only this 3.5-kilobase pair *Bam*HI restriction fragment and the vector pLC544 (Fig. 1). Neither plasmid complements the *cdc10* mutation in yeast³, which indicates that one of the two *Bam*HI sites on pYe(*CDC10*)1 is probably close to or within the *CDC10* gene. Both pYe(*CEN3*)11 and pYe35 DNAs transform *Trp*⁻ yeast to *Trp*⁺ with high efficiency³, since

Table 1 Mitotic stability in yeast of various plasmids and minichromosomes containing DNA from the centromere region of chromosome III

Plasmid	No. of individual transformants tested		Average per cent of <i>Trp</i> ⁺ or <i>Leu</i> ⁺ transformants remaining after nonselective growth	
	Strain XSB52-23C	Strain RH218	Strain XSB52-23C	Strain RH218
pYe(<i>CDC10</i>)1	5	ND	97	ND
pYe(<i>CEN3</i>)11	5	3	95	83
pYe(<i>CEN3</i>)41	2	ND	91	ND
pYe35	5	5	<1	7
pYe98F4T	5	ND	<1	ND
pYe65H3T	2	ND	<1	ND
pYe101C3T	2	ND	<1	ND
pYe(<i>CEN3</i>)21	4	ND	<1	ND
pYe(<i>ars1-ars2</i>)1	ND	8	ND	5

Yeast strains XSB52-23Ca(*cdc10 gal leu2-3 leu2-112 trp1*) and RH218a (*CUP1 gal2 mal SVC trp1*) were transformed with the above plasmid DNAs and in each case several individual transformants were isolated and grown nonselectively overnight on YPD (rich) medium. Each culture was subsequently streaked for single colonies on YPD agar, allowed to grow for 2 days at 23 °C (XSB52-23C) or 32 °C (RH218) and replica-plated onto minimal agar medium with or without tryptophan or leucine, depending on the marker carried by the plasmid. Between 40 and 100 individual clones of each original transformant were scored for plasmid loss after growth at 23 °C (XSB52-23C) or 32 °C (RH218). Preparations of media and transformation of yeast were carried out as described by Hsiao and Carbon⁹. ND, not determined.

they contain a *TRP1 ars1* vector. However, only one, pYe(*CEN3*)11, yields transformants that are all mitotically stable for the *Trp*⁺ phenotype (Table 1). When a mating was carried out between XSB52-23C/pYe(*CEN3*)11(*trp1 cdc10/TRP1*) and X2928-3D-1A(*trp1 ade1*), data similar to that for crosses 1-4 were obtained (Table 2). In 60% of the asci 2+:2- segregation was observed for the *TRP1* marker on pYe(*CEN3*)11. The minichromosome was lost entirely in 27% of the asci and in 13% was found in all four spores. Again only parental and non-parental ditype asci were obtained with respect to centromere reference markers, indicating centromere linkage of *TRP1*. No linkage of the minichromosome to chromosomes I or III was observed, since pYe(*CEN3*)11 segregated independently of the *ade1* and *cdc10* markers.

Because *trp1* yeast transformants harbouring pYe98F4T are all unstable for the *Cdc10*⁺ (and *Trp*⁺) phenotype, and those carrying pYe(*CEN3*)11 are all stable for *Trp*⁺ (Table 1), the stabilizing or centromeric DNA (*CEN3*) is most probably contained in pYe(*CEN3*)11 (and pYe(*CDC10*)1) on the 1.6-kilobase pair segment which extends from the *Bam*HI site to the end of the insert in the direction of the *leu2* locus (Fig. 1). To test this hypothesis, we recloned the 1.6-kilobase pair yeast DNA segment into the plasmid, pGT12(*LEU2 ars1*)¹¹. The DNA segment was excised from pYe(*CEN3*)11 DNA with the enzymes *Bam*HI and *Hind*III. This treatment yields a single *Bam*HI-*Hind*III restriction fragment whose *Bam*HI site is derived from the single *Bam*HI site in pYe(*CEN3*)11 and whose *Hind*III site is derived from a *Hind*III site in the vector (pLC544), located about 400 base pairs from the end of the insert of yeast DNA. The 1.6-kilobase pair segment of interest, which is thus included on a 2.0-kilobase pair *Bam*HI-*Hind*III fragment, was then inserted into plasmid pGT12, also cut with a combination of *Bam*HI and *Hind*III.

Plasmid pGT12 was constructed by inserting a 1.4-kilobase pair *Eco*RI restriction fragment containing *TRP1* and *ars1* (ref. 7) and a 2.2-kilobase pair *Pst*I fragment containing the *LEU2* gene¹¹ into the single *Eco*RI and *Pst*I sites of pBR322 (ref. 11). This plasmid also carries resistance to tetracycline. When the 2.0-kilobase pair *CEN3* fragment is inserted into pGT12 cut with a combination of *Bam*HI and *Hind*III, both Tet^R and *TRP1* expression are destroyed, but the *ars1* chromosomal

replicator is left intact. The resulting plasmid, pYe(*CEN3*)41 (Fig. 2), thus carries both *CEN3* and the replicator *ars1* in addition to the *LEU2* marker.

Plasmid pYe(*CEN3*)41 transforms strain XSB52-23C(*leu2-3 leu2-112*) to *Leu*⁺ with high frequency and is stable in the transformants (Table 1). Its behaviour in meiosis (cross 6, Table 2) is essentially the same as that described for pYe(*CDC10*)1 and pYe(*CEN3*)11 in crosses 1-5. Thus the presence of the 1.6-kilobase pair segment (*CEN3*) on a plasmid carrying a yeast chromosomal replicator is all that is required for the plasmid to behave as a chromosome in mitosis and meiosis in the majority of asci analysed.

CEN3 minichromosomes are not integrated into the yeast genome

Genetic data in crosses 1-6 confirm random assortment of *CEN3* minichromosomes with respect to chromosomes I, III, IV, and XI. The possibility that *CEN3*-containing plasmids are integrated near the centromere of one or several other yeast chromosomes was tested both biochemically and genetically. First a mating was performed between two minichromosome-bearing progeny (cross 7, Table 2). In the majority of asci in cross 7, the minichromosomes (both *TRP1*) show the 2+:2- segregation pattern, indicating that the minichromosomes frequently go to the same pole in the first meiotic division and thus do not pair with each other all the time, if at all. This segregation pattern also confirms that pYe(*CDC10*)1 is not integrated into any other single chromosome in the parents in this cross. If it were, all daughter spores should contain plasmid, because the chromosome pair into which the plasmid is integrated would duplicate in meiosis I and go to opposite poles. This point is again confirmed in cross 8 where the minichromosomes are individually marked. In at least two asci both minichromosomes went to the same pole in meiosis I (Tables 2 and 3) and were therefore both found in the same two sister spores.

In addition, biochemical evidence shown in Fig. 3 demonstrates that pYe(*CEN3*)41 is not integrated anywhere in the yeast genome. Crude yeast DNA was prepared from two germinated sister spores that contained the minichromosome

Table 2 Meiotic segregation of the minichromosomes

Genetic cross no.	Minichromosome in cross	Minichromosome marker scored	Distribution in tetrads of genetic marker on minichromosome (%)					Test for centromere linkage of marker on minichromosome			Reference centromere marker (chromosome)
			4+:0-	3+:1-	2+:2-	1+:3-	0+:4-	PD	NPD	T	
1	pYe(<i>CDC10</i>)1	<i>TRP1</i>	1 (6%)	0	10 (63%)	0	5 (31%)	2	8	0	<i>met14</i> (XI)
2	pYe(<i>CDC10</i>)1	<i>CDC10</i>	1 (8%)	0	11 (92%)	0	0	2	8	1	<i>ade1</i> (I)
3	pYe(<i>CDC10</i>)1	<i>TRP1</i>	1 (7%)	0	11 (79%)	0	2 (14%)	4	7	0	<i>met14</i> (XI)
4	pYe(<i>CDC10</i>)1	<i>TRP1</i>	4 (21%)	0	11 (58%)	0	4 (21%)	ND	ND	ND	—
5	pYe(<i>CEN3</i>)11	<i>TRP1</i>	2 (13%)	0	9 (60%)	0	4 (27%)	4	5	0	<i>ade1</i> (I)
								5	4	0	<i>cdc10</i> (III)
6	pYe(<i>CEN3</i>)41	<i>LEU2</i>	3 (14%)	3 (14%)	13 (62%)	1 (5%)	1 (5%)	7	6	0	<i>trp1</i> (IV)
								7	5	0	<i>cdc10</i> (III)
7	pYe(<i>CDC10</i>)1 × pYe(<i>CDC10</i>)1	<i>TRP1</i>	6 (35%)	0	9 (53%)	1 (6%)	1 (6%)	ND	ND	ND	—
8	pYe(<i>CDC10</i>)1 × pYe(<i>CEN3</i>)41	<i>TRP1</i>	10 (24%)	1 (2%)	31 (74%)	0	0	15	16	0	<i>met14</i> (XI)
		<i>LEU2</i>	8 (19%)	2 (5%)	24 (57%)	0	8 (19%)	10	14	0	<i>met14</i> (XI)
								17	2	0	<i>TRP1</i> (mini)

In all the above crosses the marker used to follow the minichromosome was wild type on the minichromosome and mutant in both parents. The crosses were: 1. XSB52-23Cα/pYe(*CDC10*)1(*cdc10 leu2-3 leu2-112 trp1 gal/CDC10 TRP1*) with X2928-3D-1Aa(*ade1 gal1 his2 leu1 met14 trp1 ura3*); 2. SB17Aa/pYe(*CDC10*)1 (*ade1 cdc10 leu2-3 leu2-112 met14 trp1/CDC10 TRP1*) with 6204-18Aa(*cdc10 thr4 leu2-3 leu2-112*); 3. SB1Ca/pYe(*CDC10*)1(*his2 leu1 met14 trp1/CDC10 TRP1*) with XSB52-23Cα (*cdc10 leu2-3 leu2-112 trp1 gal*); 4. SB17Ba/pYe(*CDC10*)1 (*ade1 cdc10 leu2-3 leu2-112 met14 trp1/CDC10 TRP1*) with X2928-3D-1Aa; 5. XSB52-23Cα/pYe(*CEN3*)11 with X2928-3D-1Aa; 6. XSB52-23Cα/pYe(*CEN3*)41 with X3144-1Da(*ade2 arg9 can1 his2 his6 leu2 pet8 trp1*); 7. SB14Ba/pYe(*CDC10*)1 (*ade1 his2 trp1/CDC10 TRP1*) with SB17Ba/pYe(*CDC10*)1(*ade1 cdc10 leu2-3 leu2-112 met14 trp1/CDC10 TRP1*); 8. XSB52-23Cα/pYe(*CEN3*)41 with SB17Aa/pYe(*CDC10*)1. Transformation of yeast in the presence of polyethylene glycol¹² results in a high proportion of diploid transformants (*a/a* or *α/α*)⁸. Diploids are easily recognized by the extremely low spore viability obtained when they are crossed with a haploid, and in this way were eliminated from the group of haploid transformants used in the above crosses. PD, parental ditype; NPD, nonparental ditype; T, tetratype; ND, not determined.

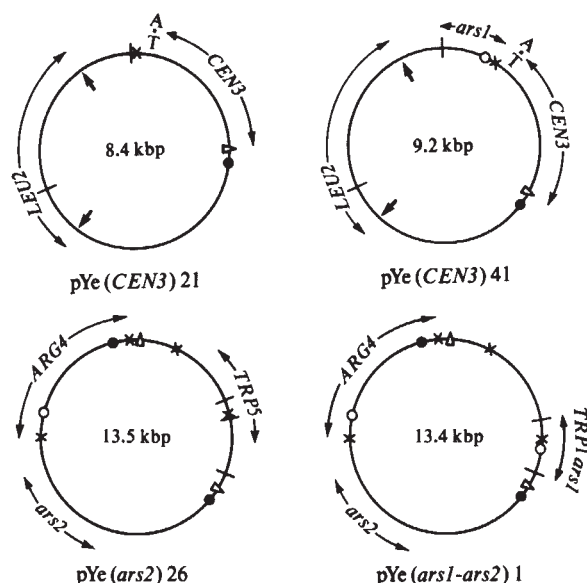


Fig. 2 Physical maps of various plasmid DNAs. The maps show the location of *EcoRI* (—+—), *HindIII* (—x—), *BamHI* (—△—), *PstI* (—*—), *SalI* (—●—) and *BglII* (—○—) sites in the DNAs and indicate approximate locations of pertinent replicators and genes. The construction and use of these plasmids is described in the text. Their sizes are indicated in kilobase pairs (kbp).

(*LEU2*) and from one of the two sisters in the same ascus that was phenotypically *Leu*⁺. This tetrad, in which the minichromosome pYe(*CEN3*)41 segregated 2+ : 2-, was obtained from cross 6 (Table 2). Figure 3 shows an autoradiograph of a Southern blot hybridization¹² in which each of the DNA preparations above was cut to completion with *SalI*, *BamHI*, or *BglIII*. There is a single restriction site for each of these enzymes in the vector portion of pYe(*CEN3*)41 or between the vector and the yeast DNA insert (Fig. 2). In Fig. 3, lanes with DNA from minichromosome-containing progeny (lanes *b* and *c*) show a single band of hybridization to the radioactive probe, [³²P]pBR322 DNA. This band has the same mobility regardless of the enzyme used to cut the DNAs, and the mobility is identical to that of linear pYe(*CEN3*)41 generated in the presence of a plasmid-free yeast DNA preparation (lanes *d*) with *BamHI* or *BglIII*. The absence of any hybridization bands of sizes other than linear pYe(*CEN3*)41 suggests that this minichromosome is not integrated into yeast genomic DNA. We have not yet attempted to integrate any of the pYe(*CEN3*) plasmids into genomic DNA. Such an event would be expected to lead to dicentric chromosomes and probable mitotic instability¹³.

Functional chromosomes require both centromeric DNA and a chromosomal replicator

All the minichromosomes studied and described above contain both *CEN3* and the chromosomal replicator *ars1*. The need for such a replicator with regard to *CEN3* function was tested by inserting the 2.0-kilobase pair *BamHI*–*HindIII* restriction fragment carrying *CEN3* into the plasmid pGT6. Plasmid pGT6 is pBR322 (Tet^r, Amp^r) with a 2.2-kilobase pair fragment carrying the yeast *LEU2* gene inserted into its single *PstI* site¹¹. This plasmid also carries resistance to tetracycline, but not resistance to ampicillin. The 2.0-kilobase pair *BamHI*–*HindIII* *CEN3* fragment was ligated into *BamHI*–*HindIII* cut pGT6 DNA, resulting in the inactivation of Tet^r and yielding the plasmid, pYe(*CEN3*)21, whose structure is shown in Fig. 2.

Somewhat surprisingly, pYe(*CEN3*)21 DNA which contains *CEN3*, but lacks a chromosomal replicator sequence (Fig. 2), also transforms XSB52-23C to *Leu*⁺ with a frequency as high as does pYe(*CEN3*)41 DNA. Plasmid pYe(*CEN3*)21 is very unstable mitotically, however (Table 1). When grown under

selective conditions in minimal liquid medium without leucine, the doubling time for transformants of pYe(*CEN3*)21 is about 12 hours, as compared to 4 hours for those transformants carrying pYe(*CEN3*)41. In log phase under selective conditions greater than 95% of the cells have segregated pYe(*CEN3*)21, whereas no segregation of pYe(*CEN3*)41 is detected under the same conditions. In general, more than 50% of yeast cells, originally transformed with plasmids carrying chromosomal replicators such as *ars1* (near *TRP1*) or *ars2* (near *ARG4*), lose the plasmid while being grown selectively^{7,9}. The high frequency of transformation and high mitotic instability exhibited by pYe(*CEN3*)21 in yeast is perhaps most easily explained by postulating the presence of a weak or partial replication origin on the 1.6-kilobase pair (*CEN3*-containing) segment that permits only enough replication for the plasmid to be transferred to a few daughter cells in the population. It is not known whether this origin plays a role in centromere function.

Plasmids containing two chromosomal replicators are mitotically unstable

Since pYe(*CEN3*)21 DNA does transform yeast with high efficiency in the absence of a chromosomal replicator and seems to have a weak origin of replication of its own, we tested the possibility that any two chromosomal replicators on a plasmid might give mitotic stability to that plasmid in yeast. This notion has been addressed previously by Stinchcomb *et al.*⁷, who looked at the behaviour of multimeric forms of *ars1* and found that yeast cultures transformed with monomer, dimer, and trimer *YRp7*(*TRP1 ars1*) were equally unstable mitotically and had identical growth rates.

We have put the *ars1* replicator together on a plasmid with *ars2* (a chromosomal replicator near *ARG4*)⁹ in the following way. The plasmid pYe(*ars2*)26 (Fig. 2) contains the yeast *ARG4* and *TRP5* genes and the *ars2* replicator (C. L. Hsiao and J.C., manuscript in preparation). Plasmid pYe(*ars1-ars2*)1 was constructed by treating pYe(*ars2*)26 DNA with *EcoRI* endonuclease and inserting the 1.4-kilobase pair fragment carrying *Trp1* and *ars1* (ref. 7) into the plasmid DNA, thereby partially deleting the *TRP5* gene and replacing it with *TRP1* and *ars1* (Fig. 2). When *trp1* yeast was transformed to *Trp*⁺ with pYe(*ars1-ars2*)1 DNA, transformants were as unstable mitotically for the *Trp*⁺ phenotype as those transformed with pYe35 which carries only *ars1* (Table 1). In the same experiment, pYe(*CEN3*)11 showed less than 18% loss of the *Trp*⁺ phenotype. In addition, the *ars1* replicator segment is in opposite orientations with respect to *CEN3* in pYe(*CDC10*)1 and pYe(*CEN3*)41, and both minichromosomes are equally stable. We conclude that two chromosomal replicators together do not convey mitotic stability upon a plasmid and that *CEN3* is unique

Table 3 Genetic analysis of mating between two haploid parents each carrying an individually marked minichromosome (cross 8)

Distribution in tetrads of markers on minichromosomes		No. of tetrads showing segregation pattern (% of total)
<i>TRP1</i> (pYe(<i>CDC10</i>)1)	<i>LEU2</i> (pYe(<i>CEN3</i>)41)	
4+ : 0-	4+ : 0-	3 (7%)
4+ : 0-	2+ : 2-	4 (10%)
4+ : 0-	0+ : 4-	3 (7%)
3+ : 1-	2+ : 2-	1 (2%)
2+ : 2-	4+ : 0-	5 (12%)
2+ : 2-	3+ : 1-	2 (5%)
2+ : 2-	2+ : 2-	19 (45%)
2+ : 2-	0+ : 4-	5 (12%)
Total no. of asci analysed		42 (100%)

Strains in cross 8 are described in Table 2, where the meiotic segregation patterns of the minichromosomes are summarized. Among the 19 asci where both minichromosomes segregated 2+ : 2-, both went to the same pole in the first meiotic division in 2 asci and to opposite poles in 17 asci.

in its stabilizing effect. This conclusion is further verified by the observation that *CEN3*, when isolated on a 5.1-kilobase pair *EcoRI* fragment derived from plasmid pYe46B2 (Fig. 1) and inserted into the *EcoRI* sites at the *TRP5* locus on pYe(*ars2*)26 (Fig. 2), mitotically stabilizes this *ARG4 ars2* plasmid for the Arg⁺ phenotype (C. L. Hsiao and J.C., unpublished result).

Minichromosomes do not always pair in the first meiotic division

The absence of asci with two viable and two nonviable sister spores in crosses 1–6 indicates that the minichromosomes do not pair at the first meiotic division with any of the 17 yeast chromosomes. Cross 8 (Tables 2 and 3) was performed with two strains, each harbouring a *CEN3 ars1* plasmid marked with either *LEU2* or *TRP1*. Both parents in cross 8 have mutations at both *leu2* and *trp1*, therefore the behaviour of each minichromosome can easily be followed individually. A detailed analysis of this cross is given in Table 3, from which the following conclusions can be drawn. Each plasmid individually exhibited the 2+:2– segregation pattern in more than 60% of the tetrads

examined. In the 19 asci where both plasmids segregated 2+:2–, the minichromosomes went to opposite poles in the first meiotic division in 17 asci, but they did assort to the same pole in 2 asci. These numbers support the notion that the minichromosomes do not always pair, but there is a strong preference for them to go to opposite poles in the first division. Otherwise, they seem to segregate randomly and independently with respect to one another, and all combinations of 4+:0–, 2+:2–, and 0+:4– are seen (Table 3). For example, both minichromosomes were found in all four spores in three tetrads; in five tetrads pYe(*CEN3*)41 was found in all four spores, but pYe(*CDC10*)1 segregated 2+:2–; in three asci pYe(*CDC10*)1 was in all four spores, but pYe(*CEN3*)41 was completely lost. Thus two minichromosomes together in the same sporulating diploid show the same meiotic behaviour as either one exhibits alone.

Discussion

We have identified a small DNA element (*CEN3*) from *S. cerevisiae* chromosome III that when present on a plasmid with a chromosomal replicator (*ars1* or *ars2*) allows the plasmid to behave as a functional chromosome in yeast. Minichromosomes containing *CEN3* are stable in mitosis, and in most cases segregate as chromosomes in the first meiotic division and are thus found in the two sister progeny of the second meiotic division. The *CEN3* element is therefore capable of controlling copy number, thereby ensuring proper meiotic segregation. In approximately 15% of the tetrads analysed in crosses involving *CEN3* plasmids, the minichromosome is lost completely. Generally, in yeast strains that are aneuploid for one or more chromosomes, the extra chromosomes are also somewhat unstable¹⁴. Tetrads in which a *CEN3* minichromosome segregates 1+:3– are rare, therefore the minichromosome is more stable through the second meiotic division than in the first.

In approximately 20% of the tetrads analysed, the minichromosome is found in all four spores after meiosis. This may be a result of selective diploidization of the *CEN3* plasmid or could indicate a loss of control of minichromosome replication. Inability of minichromosomes to pair may cause a certain instability in their attachment or involvement in the meiotic apparatus. Thus control over plasmid replication may be eliminated in some cases resulting in multiple copies that are distributed to all four spores. The 4+:0– tetrads are not a separate class, since the same 4+:0–, 2+:2–, 0+:4– patterns are exhibited by the progeny after further matings. Moreover, data from cross 8 (Tables 2 and 3) show that one minichromosome can segregate 2+:2– while another in the same meiotic event is distributed 4+:0–.

The genetic data show that minichromosomes do not pair with any of the normal yeast chromosomes, since asci containing two non-viable sister spores are not seen. However, two minichromosomes in the same diploid exhibit a strong preference to migrate to opposite poles in the first meiotic division. To determine exactly how much DNA, or which regions of DNA are involved in pairing of chromosomes at the first meiotic division, increasingly larger segments of DNA may be added *in vitro* to either or both ends of the *CEN3* segment on a plasmid. Pairing could then be assayed by genetic analysis.

Because plasmid pYe98F4T complements the *cdc10* mutation in yeast and plasmids pYe(*CEN3*)11 and pYe35 do not (Fig. 1), we have concluded that the *CDC10* gene is probably located in the region of the single *Bam*HI site in pYe98F4T. The *cdc10* locus had previously been placed directly next to the centromere of chromosome III (refs 15–17). The data presented here indicate that *cdc10* is located about 3 kilobase pairs from the centromere on the right arm of chromosome III. There are approximately 25 kilobase pairs of DNA between the *LEU2* and *CDC10* genes³. The genetic map distance between *leu2* and centromere is about 8 cM (refs 3, 16 and 17). Thus there is an average of about 3 kilobase pairs cM^{–1} from *leu2* across the centromere to *cdc10*. This number is close to the 2.7 kilobase pairs cM^{–1} determined by Strathern *et al.*¹⁸ for very large

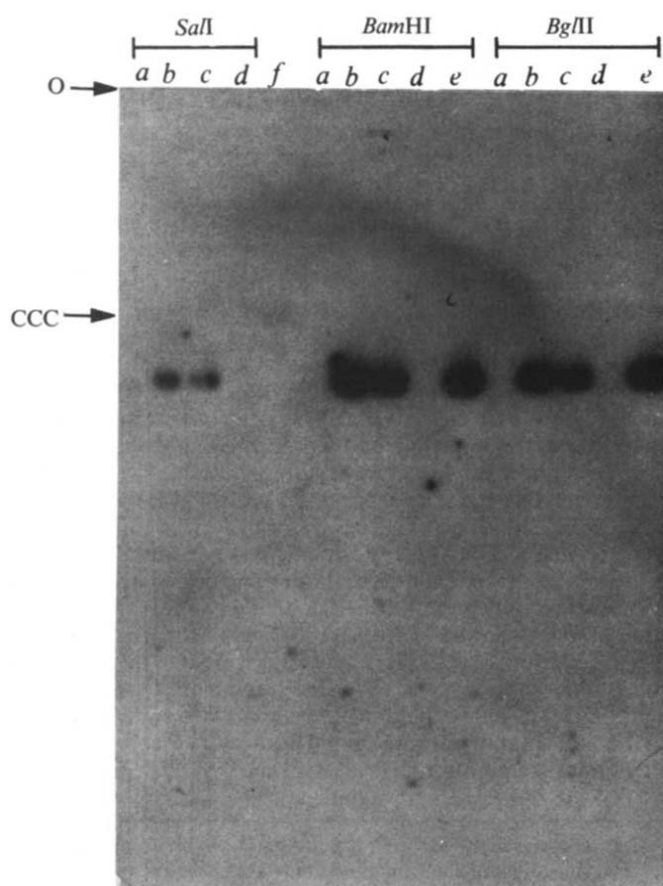


Fig. 3 Southern blot hybridization autoradiograph of total yeast DNAs from pYe(*CEN3*)41-containing strains with [³²P]pBR322 DNA as probe. Crude yeast DNA was prepared as described by Stinchcomb *et al.*⁷ from one *Leu*[–] progeny (lanes a) and two *Leu*⁺ progeny (lanes b and c) of a single 2+:2– ascus in cross 6 and from a *Leu*[–] progeny of a 3+:1– ascus in the same cross (lanes d). Approximately 2 µg of each DNA preparation was digested with *Bam*HI, *Sal*I, or *Bgl*II and electrophoresed in 0.75% agarose. Other lanes on the gel contained 2 ng of pYe(*CEN3*)41 DNA mixed with 2 µg of crude *Leu*[–] yeast DNA, either undigested (lane f) or digested (lanes e) with restriction enzyme. Blot hybridization was carried out according to Southern¹², with the following modifications. The fragments were transferred from the gel to nitrocellulose as described by Wahl *et al.*²¹ and hybridization conditions were those of Cameron *et al.*²². The probe was pBR322 DNA labelled *in vitro* by nick translation²³ as described by Chinault and Carbon¹. The origin (O) and position of covalently-closed circular pYe(*CEN3*)41 DNA (CCC) are indicated on the autoradiograph.

distances on the circular chromosome III, indicating that the centromere does not appreciably distort recombination frequencies in the *leu2-cdc10* region.

We have shown previously that within the limits of a standard Southern blot hybridization, plasmid pYe(*CDC10*)1 contains only unique DNA³. By this same criterion, all the DNA between *leu2* and *cdc10* is unique DNA¹. Work in progress is expected to define further the size of the centromeric DNA segment and its nucleotide sequence. Whether centromeric DNA sequences are unique for each chromosome may eventually be determined by sequencing other centromeres obtained in the same way.

The small circular chromosomes we have constructed around the *CEN3* element have many properties that mimic those of the much larger parental chromosomes. They are stable both in mitosis and through the first and second divisions of meiosis, probably because *CEN3* is providing an attachment site for spindle structures, a site that may be unique for each chromosome. A chromosomal replicator must accompany the *CEN3* segment to permit proper centromere function, but the replicator alone, or a combination of chromosomal replicators, do not stabilize a plasmid. Therefore the minichromosomes, like the larger chromosomes, require both an attachment site and one or more replicators. Unlike the parental chromosomes, the small circular chromosomes do not always pair, but, as is the case in sporulating aneuploid yeast strains, absence of pairing does not prevent the chromosomes from segregating in meiosis. Although we have no evidence that the weak replicator in the *CEN3* region is involved in centromere function, it is intriguing to speculate that it may be a specialized replicator for that region, perhaps serving to drive sister chromatids apart in a final burst of DNA synthesis, as suggested by DuPraw¹⁹.

The *CEN3* minichromosomes are excellent probes for the study of events in mitosis and meiosis, particularly with regard to the protein-nucleic acid interactions occurring between the centromere and the mitotic and meiotic spindle structures. Minichromosomes with a functional centromere will be useful as well for studies of chromatin structure and the organization of DNA-associated proteins in the centromere region.

Finally, the mitotic stability of *CEN3* plasmids make them especially suitable cloning vehicles. Studies of expression of cloned genes on minichromosomes would not be complicated by the variable mitotic stability displayed by other vectors constructed for cloning in yeast. The functional centromeric sequence, *CEN3*, is not altered by propagation in *E. coli*, since all the *CEN3* minichromosomes were originally isolated in that bacterium before being put into yeast, and the absence of any gross alterations in DNA sequence in cloned segments from around the *CEN3* region has been previously confirmed by Southern blot hybridizations to restriction digests of total yeast genomic DNA^{1,3}. It will also be of considerable interest to determine if the yeast centromere will function when introduced into other cell types on the appropriate self-replicating DNA vectors.

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A low resolution structure for the histone core of the nucleosome

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Image reconstruction to 22 Å resolution of the histone octamer (H3)₂(H4)₂(H2A)₂(H2B)₂ shows it to have a 2-fold axis of symmetry, and the overall shape of a left-handed helical spool on which to wind about two turns of a flat superhelix of DNA in the nucleosome. From this structure and the results of various cross-linking studies, we have deduced the arrangement of the individual histones. We propose that the (H3)₂(H4)₂ tetramer forms a dislocated disk which defines the central turn of DNA, while the two H2A-H2B dimers lie one on each face, each associated with about one half a turn.

THE fundamental unit of chromatin structure is the nucleosome¹ which consists of a stretch of DNA wound about a histone core. With the knowledge that the arginine-rich histones H3 and H4 existed in solution as tetramers^{2,3}, the lysine-rich histones H2A and H2B as dimers^{2,4}, and that the four histones occur in chromatin in equimolar amounts, Kornberg⁵ proposed that the core of the nucleosome was a histone octamer of composition (H3)₂(H4)₂(H2A)₂(H2B)₂. Octamers of histones in

chromatin were demonstrated by chemical cross-linking, and the octamer was also shown able to exist free in solution in 2 M NaCl, which displaces the DNA^{6,7}. The octamer in 2 M NaCl was later characterized by hydrodynamic and other methods⁸⁻¹⁰.

While attempting to crystallize the histone octamer we have produced regular fibres at high ionic strength, and have used electron microscopy and the image reconstruction method^{11,12} to produce a low resolution three-dimensional density map of

the octamer. It has 2-fold symmetry. The nature of the octamer structure suggests its orientation relative to that of the super-helix of DNA in the nucleosome, corresponding with that demonstrated by neutron diffraction studies of nucleosome core particle crystals¹³. We have used the results of Mirzabekov *et al.*¹⁴ on the chemical cross-linking of histones to nucleosome core DNA, and the information on histone-histone proximities given by cross-linking, to assign regions of density in the octamer map to particular histones. This constitutes a proposal for the spatial arrangement of histones in the nucleosome core, from which it can be seen that the (H3)₂(H4)₂ tetramer defines the basic fold of the nucleosome DNA and the H2A-H2B dimers continue it.

Preparation and electron microscopy of histone octamer tubes

The histone octamer was prepared from rat liver as described earlier⁸, in 2 M NaCl-100 mM Tris Cl, pH 7.0, or 2 M NaCl-137 mM sodium borate, pH 9.0. When a solution of the octamer is brought to >40% saturation with ammonium sulphate at 4 °C, keeping the NaCl and buffer concentrations constant (final concentration of octamer 2 mg ml⁻¹), a precipitate forms over a period of days that contains all four histones in their original equimolar amounts. Electron micrographs of negatively stained samples of the precipitate fixed with glutaraldehyde show sheaf-like aggregates of a dozen or so hollow tubular structures, of external diameter about 300 Å and several micrometres long. If fixing is omitted, the tubes break down to short lengths, some so short that they lie on the grid end-on to give an annular image (Fig. 1a arrow). Specimens more suitable for image analysis may be obtained by adding the stain to the precipitate before applying it to the grid. This dissociates the sheaves but maintains the length of the individual tubes (Fig. 1b, c). Essentially identical tubes were grown using histone octamer from turkey erythrocytes and beef kidney.

The state of aggregation of the histones in conditions where tubes form was checked by cross-linking with dimethyl suberimide^{6,15} and shown to be octameric, the time course being similar to that described previously for the free octamer in 2 M NaCl¹⁶; as expected there is also some evidence of octamer-octamer cross-linking. (Because cross-linking of amino groups cannot be carried out in ammonium sulphate, we used instead dipotassium hydrogen phosphate (0.5M)-potassium pyrophosphate (0.615M) which also promotes aggregation into tubes [B. Rushton, J.T.F. & A.K., unpublished results].)

Moreover, comparison of cross-linked dimers at comparable stages of cross-linking shows that the arrangement of histones in the octamer is essentially the same for the octamer in the tubes, the octamer free in solution, and the octamer in the nucleosome; these dimers are those reported previously in the nucleosome^{6,7} and H3-H2B. For the tubes, the same dimers are formed at roughly the same relative rates and are present in similar relative amounts, the main differences being consistent with unmasking of a higher proportion of amino groups in H3 and H4 than in H2A and H2B on going from the nucleosome to the octamer in high salt. We therefore believe that extrapolation from the model for the octamer present in the tubes (Fig. 4) to the octameric core of the nucleosome (Fig. 5) is valid.

Image analysis and 3-dimensional reconstruction

The transverse striations, of spacing about 65 Å, across the hollow tubes indicate that they are built from rings of (octamer) units. The annular images are evidently short stacks of rings seen end-on, and sometimes a regular series of protuberances can be seen around the circumference. The number of units per ring cannot be counted unequivocally, but is in the range 10-12. The number can be estimated by viewing the images of the long tubes obliquely from the bottom of the page. At this angle, the units can be seen to lie on long-pitch helices. The number of parallel helices on one side of the tube appears to be five rather than six indicating 10 units per ring. A more reliable estimate is obtained by close inspection of the edge-on views in the long tubes: the succession of superposition patterns seen—and such patterns are not as sensitive to distortions as are quantitative analyses—is characteristic of 10 units per ring. The value 10 was confirmed by the Fourier analysis described below. Another class of tube was occasionally found of slightly larger diameter, containing 11 units per ring, but they were less well ordered than the 10-fold tubes and gave poorer maps.

We have investigated the structure of the octamer tubes by Fourier transform techniques^{11,12}, using computer programs developed for correlating helical particles¹⁷. Optical transforms were used initially to select well ordered particles, then the best were processed by computer to give phase information for a quantitative assay of the state of specimen preservation, and again from among these were selected a smaller set of particles which correlated well with one another, after allowance was made for their different orientations. The data from a final set

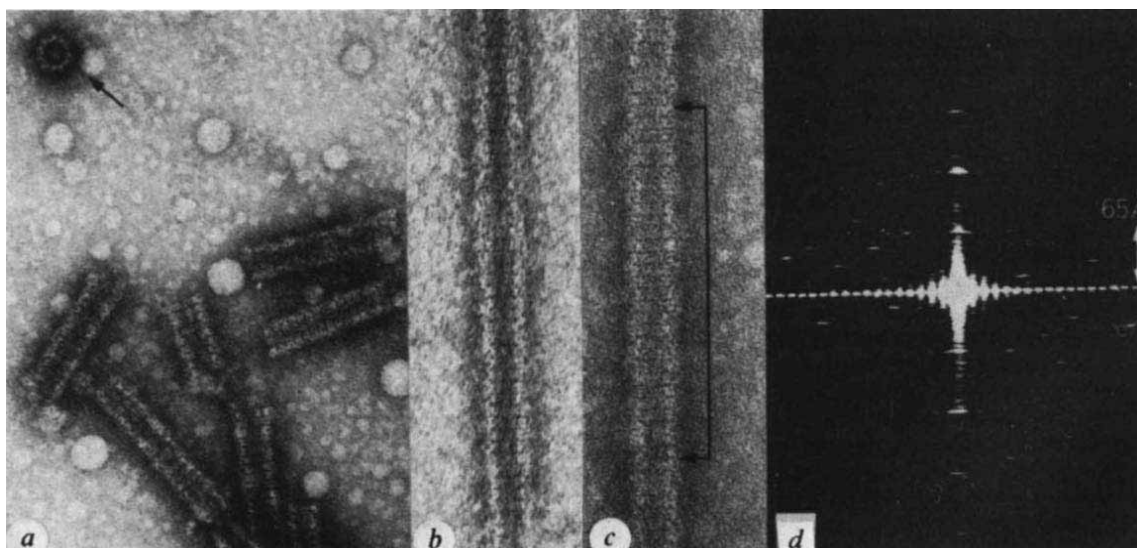


Fig. 1 Electron micrographs of tubes of histone octamers, negatively stained with 0.1% uranyl acetate; in *a*, the stain was applied after the specimen was applied to the grid, and in *b* and *c*, beforehand. Oblique helical lattice lines of pitch about 280 Å can be seen clearly in *b*, in which the contrast of one side of the particle dominates. *d*, Optical diffraction pattern of the length marked in *c*; the first meridional reflection is at an axial spacing of 65 Å. The strong second order of this reflection indicates that the projected density in the rings is roughly divided into two layers, as is evident in the images themselves. Magnifications: *a*, 175,000×; *b*, 236,000×; *c*, 242,000×.

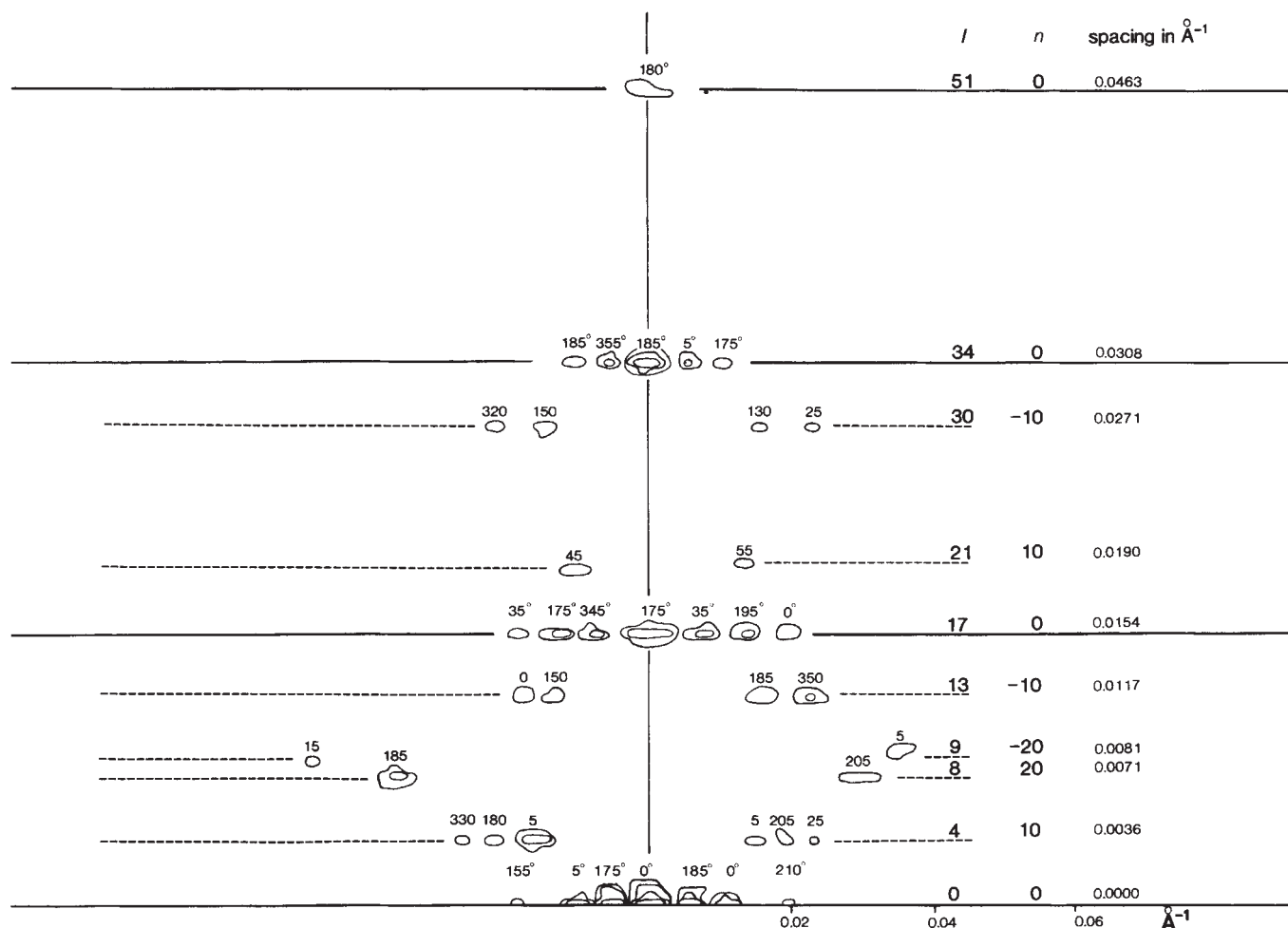


Fig. 2 A map of the amplitudes and phases in the computer Fourier transform of the octamer tube in Fig. 1c. The image was densitometered on a raster with a spacing equivalent to about 2.5 Å. The layer lines are marked by an index l corresponding to the simplest rational approximation to the helical repeat. The helical selection rule for the diffraction pattern is $n = 10n'$ where n' is given by $l = 4n' + 17m$; the orders n of the Bessel functions on each layer line are indicated. Because the rotational symmetry is of even order (10-fold), the phases of corresponding peaks on left and right sides should ideally be the same, and the discrepancy between them is a measure of the state of preservation of the specimen. The presence of radial dyads was tested for by searching for the position of putative dyads in the image which make the phases as close as possible to 0° or 180° . The lowest value of the (weighted) residuals found (corresponding to the Q-type of residual (of ref. 17) for the three best particles were individually 41° , 38° and 18° , and 31° for the average, showing that dyads are present. The best particles were found by examining the correlation between the two sides of their transform (near and far sides of the particle) and the correlation between different particles. During this analysis, a radial scale factor is computed to help compensate for variations in magnification and particle shrinkage during drying; most such scale factors varied by less than 10% from the average. When there is no overlap of different helical contributions on the same layer line, as here, each Fourier transform yields two independent data sets, corresponding to the near and far sides of the particles. Separate radial scale factors were used for the two sides to allow for differential shrinkage, the J_0 contributions being omitted for the determination of the scaling factor, but apportioned afterwards, one half to each side.

were combined to calculate Fourier components for a three-dimensional synthesis.

An optical diffraction pattern and computer transform of one of the best tubes are shown in Figs 1d and 2 respectively. The axial reflection on a layer line of spacing 65 Å corresponds to the thickness of a ring. Off-meridional intensity occurs on a layer line of spacing about 280 Å, and corresponds to helical lines of pitch 280 Å running obliquely across the particles (Fig. 1b). The absolute hand (left) of this helical family, and hence of the whole structure, was found from electron micrographs of shadowed specimens. The relative rotation of successive rings is reflected in the ratio of the spacings of the 280 Å and 65 Å layer lines, which varies between 4.0 and 4.4 for different particles and different regions along the same particle. An average of 4.25 ($=\frac{17}{4}$), corresponding to a rotation of $2\pi/10 \times (4/17)$ between successive rings, was adopted as the simplest rational approximation for numbering the layer lines in Fig. 2.

Analysis of the computer transforms of images of the tubes showed that, to the resolution of the micrographs, the histone octamer itself possesses a 2-fold axis of symmetry. Transforms were correlated with each other¹⁷ to bring all the particles into a common registration, to permit data averaging. During this

fitting procedure, a phase residual is calculated which gives a measure of how well correlated any two particles are. No significant differences in the residuals were found between the best fits of particles either way up, showing that the tubes were non-polar and so possessed radial dyad axes. The final map (Fig. 3), comprising the average data from three particles, has a nominal resolution of about 20 Å in both axial and radial directions, but all contributions at less than about 30 Å are weak. The three particles chosen were those that correlated best with each other, and also individually showed equally good preservation in their near and far sides.

Description of the structure

The map of the octamer tube reconstructed from the averaged data with imposed 2-fold symmetry is presented in Fig. 3. The rings of the tube are divided into ten distinct units, each presumably representing an octamer; the division between rings is also clear, and thus, except for small boundary regions, the density corresponding to one octamer can be unequivocally dissected from the map. A model of this density is shown in Figs 4a and 7a: the contour cutoff in constructing the model was

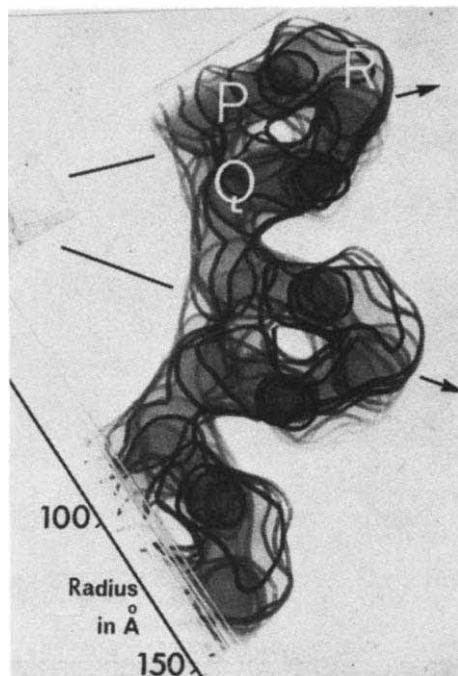


Fig. 3 Three-dimensional reconstruction of the histone octamer tube, plotted as sections through the map. The region shown corresponds to $2\frac{1}{2}$ octamers, that is a 90° sector of the tube of height $65 \text{ \AA}$, and is viewed in a direction tilted slightly away from the tube axis. The lowest contour level has been chosen to give the appropriate hydrated molecular volume of the octamer. The dyad axes of symmetry passing through the octamers are marked by arrows.

chosen to give a hydrated molecular volume of $137,000 \text{ \AA}^3$, taking $M_r = 110,000$ and assuming a density for hydrated protein of $0.8 \text{ daltons \AA}^{-3}$ (ref.18).

Figure 4a shows the wedge-shape appearance of the model, whereas Fig. 7a shows that at its broadest aspect the particle has a roughly circular outline of approximate diameter $70 \text{ \AA}$. At right angles to this the particle is $56 \text{ \AA}$ at its thickest point. These are suitable dimensions to form the histone core of a nucleosome core particle of external diameter $110 \text{ \AA}$ and thickness $55 \text{ \AA}$ (ref. 19): subtracting the DNA from the outside would leave a cylindrical inner space of about $70 \times 55 \text{ \AA}$.

The correspondence goes further. The nucleosome core is a wedge-shaped particle, of bipartite character¹⁹, and the octamer also has a similar wedge shape, split into two and twisted somewhat about its dyad axis. This is best seen in Fig. 3 where the wedge is viewed approximately sideways on. The two ridges at the thick end, marked P and Q, slope up in an opposite direction to the ridge at the thin end, marked R, so that they, together with the ridges on the sides of the wedge, form a rough helical ramp, of pitch about $25 \text{ \AA}$, whose course is marked in Fig. 4b. The ridges forming the ramp are rather broken up, and there is some variation in individual image reconstructions, but the continuity is not in doubt. The sense of the ramp is left-handed, as expected if the DNA superhelix is to wind around the octamer.

The octamer shape revealed here resembles that found in the density map of the protein moiety of the nucleosome core, produced from a neutron diffraction study of crystals of nucleosome core particles, using the method of contrast variation (Fig. 2c of ref.13). That study also gave a map of the DNA, and hence the orientation of the 'wedge' of $1\frac{3}{4}$ turns of DNA¹⁹ relative to the wedge formed by the histone core. This orientation is the same as that shown in Fig. 4b. It is thus the octamer that determines the architecture of the nucleosome. The 2-fold symmetry of the DNA superhelix allows it to interact with its octameric histone core along a common twofold axis.

Note that the map identifies the stain-excluding regions, so that the model presumably depicts the hydrophilic surfaces of

the particle, which are exposed to the negative stain, and is thus dependent on the way in which the stain actually interacts with (and possibly distorts) the surface of the histone molecules and penetrates the gaps between them. At low resolution, the individual histone molecules are not resolved, yet our map shows protuberances presumably corresponding to exposed portions of the individual molecules making up the oligomer, and channels corresponding to the spaces between them.

Clearly the octamer has fairly large cavities in it accessible to the negative stain. These run irregularly through the structure to a hollow centre, and, together with the overall flat shape, could surely account for the anomalous frictional ratio of about 1.3, found from hydrodynamic observations⁸, even without a contribution from any flexible N-terminal tails²⁰.

When a superhelix (representing the DNA) is wound smoothly at a mean radius of about $45 \text{ \AA}$ on the helical spool formed by the octamer, as in Fig. 5, it fits snugly at the thick and thin ends of the wedge, but does not hug the sides so well. Although this might be an artefact, it could reflect true disorder arising from flexible N-terminal regions of the histones, which might be lost to view in the stain and would certainly be lost in the averaging procedure. The differential fit may be related to the differential cutting along the DNA of the nucleosome core shown by DNase I. We noted earlier that the regions of low cutting frequency lie at two diametrically opposite areas, corresponding to the 'sites' -4 and $+4$ on one side, and -1 and $+1$ on the other (Fig. 8 of ref. 19). It is likely that this increased protection is associated with tighter contact with the histone core at the two ends of the wedge. Indeed, the distribution of diffuse intensity in X-ray diffraction patterns of the nucleosome core crystals shows that the DNA on these two 'ends' of the nucleosome core is held more rigidly than that at the 'sides' (J.T.F. and A.K., unpublished results). A stiff molecule like DNA wound neatly on to a spool need not have equal contact all along its length; it need only be fixed at a few points and the bending stiffness will do the rest.

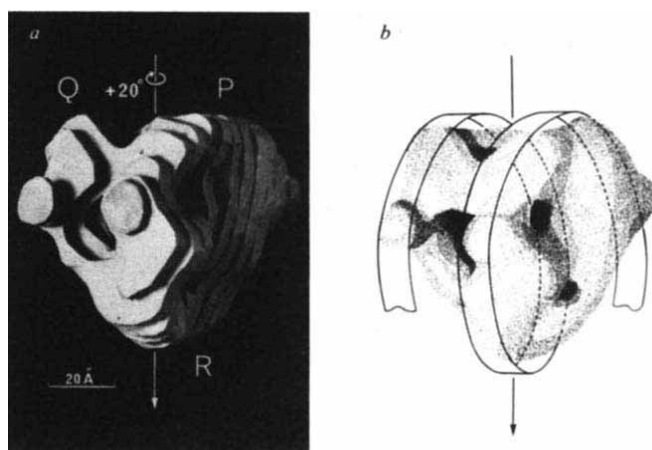


Fig. 4 *a*, Model of histone octamer obtained by cutting out solid sections of the map in Fig. 3. The dyad axis is marked by an arrow, and the view is at an angle of about 20° to that in Fig. 3, as can be seen from the inclination of the sections. *b*, A drawing of *a* showing how the ridges on the periphery of the octamer model form a more or less continuous helical ramp of external diameter about $70 \text{ \AA}$ and pitch about $27 \text{ \AA}$, on which about $1\frac{3}{4}$ turns of a superhelix of DNA of the appropriate dimensions¹⁹ could be wound. In a further reconstruction, not included here, there is a lower density at one of the ends of the ridges P and Q (between the marks H4¹ and H2B¹ in Fig. 5a) but the continuity of the ramp is clear.

Assignment of histones to regions of the map

We have used histone-DNA cross-linking results, which map the sites of interaction of histones relative to the 5'-end of a DNA strand, and information on histone-histone proximities from cross-linking studies, to assign individual histones to particular regions of the density map. Our main assumption is

that the histones are too small to be multi-domain proteins, although they could still have flexible N-termini. Others^{14,21,22} have suggested three-dimensional arrangements of histones within the nucleosome core, using similar chemical evidence, but an interpretation based on such results alone is ambiguous because neither type of cross-linking study distinguishes between the two copies of each histone present. Our three-dimensional density map restricts the number of possibilities. Thus, for example, the ridge R (Fig. 4a) which lies in the region of DNA sites -1 to +1 (Fig. 5a) is too narrow to accommodate more than one thickness of a histone molecule, and other models are not compatible with this feature. The proposed arrangement is the only one we can find that both fits the major cross-linking data and is compatible with the shape of the octamer.

Histone-DNA cross-linking

The points of contact of histones along the DNA strands determined by histone-DNA cross-linking studies¹⁴ are not sufficient to fix the spatial arrangement of the histones in the nucleosome core. Using the three-dimensional structure in Fig. 4 we have distinguished between the two copies of each histone as shown in Fig. 5. If the two copies of each histone are designated by superscripts 1 and 2, then the results of Mirzabekov *et al.*¹⁴ may now be written more specifically as shown in Fig. 6. Thus, for example, the H3 that makes contact with the DNA 75 bases from the 5' end is H3¹, whereas the H3 that lies close to the 3' end (see also ref. 23) is H3².

Note that we do not always assume that the pattern of histone-DNA cross-links observed by Mirzabekov *et al.*¹⁴ directly reflects the linear order of histones along the DNA. Even if the major contact of a histone is with a particular DNA segment, it is entirely possible (because the distance between the two superhelical turns is only about 28 Å, that is, roughly the dimensions of a histone molecule) that a histone may also make a less frequent, or minor, contact with the turn of DNA above or below it. Thus, to reconcile our model with the finding¹⁴ of a contact between H2A and the DNA 75 bases from the 5'-end (between sites 0 and 1 in Fig. 5), we have assigned this contact to H2A¹, which also lies close to the DNA turn above, near the segment 0-20 bases from the end (sites -7 and -6). No histone-DNA cross-link corresponding to this contact is observed¹⁴, but an H2A molecule is required there by symmetry, because of the contact at 135 base pairs (between sites 6 and 7); the absence of a cross-link need not be significant and might arise through lack of suitably disposed lysine side chains in the region of contact.

Recently²⁴ Mirzabekov and his colleagues have refined their results and have assigned particular histone-DNA contacts to individual DNA strands. However, the weak spots on gels identified in that work need not arise from oscillations of linkages between strands as suggested and can be accommodated into our spatial arrangement with the reasonable assumption that a particular histone can cross-link to either one of the neighbouring DNA strands of the double helix. We attempt here only to associate a histone with a particular region of the DNA double helix, without regard to strand.

If the DNA superhelix in the model (Fig. 5) were unfolded, the linear arrangement of the histones along the DNA would be:

$$\text{H2A}^1\text{-H2B}^1\text{-H4}^1\text{-H3}^1\text{-H3}^2\text{-H4}^2\text{-H2B}^2\text{-H2A}^2$$

(site -7 → site +7)

This might seem to be at odds with the contacts broken when the nucleosome unfolds at low ionic strength, or in urea or urea/2M NaCl²⁵⁻²⁷. However, in the first case²⁵, although two particular residues (Cys 110 in the C-terminal region of the H3s) move apart, the basic N-terminal regions of the proteins may not behave in the same way; and in the other cases^{26,27}, loss of particular cross-links generated by 'zero-length reagents' could result from only small local perturbations, because these reagents require very precise contacts and juxtapositions for cross-linking.

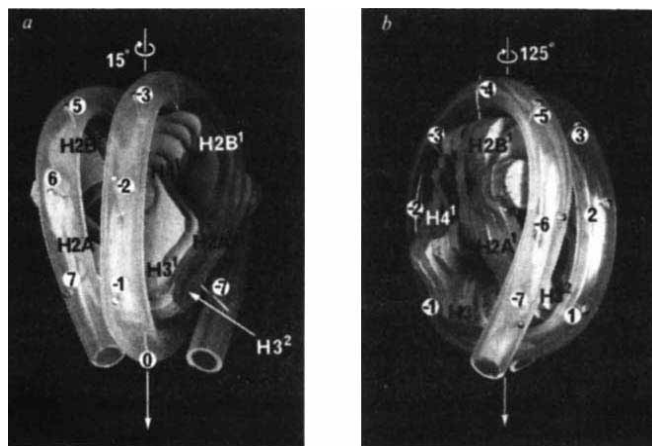


Fig. 5 a, The histone octamer structure with two turns of a DNA superhelix wound on it. The assignment of the individual histones to various parts or features of the model is described in the text. (For clarity the DNA double helix is represented by a plastic tube of somewhat smaller diameter than it would actually have.) Distances along the DNA are indicated by numbers -7 to +7 (taking the dyad axis as origin), to mark the 14 repeats of the double helix contained in the 146 base pairs of DNA of the nucleosome core. The positions of the numbers correspond approximately to the sites of attack, that is local maxima in the frequency of cutting by the enzyme DNase I (compare with Fig. 8b of ref. 19). The superscripts in the numbering of the histone molecules follows the direction of ordering of the DNA sites, so that the helical ramp of the octamer is formed by the histones in the order H2A¹-H2B¹-H4¹-H3¹-H3²-H4²-H2B²-H2A². b, Another view of a, rotated further by 110°; the direction of view is similar to that in Fig. 7a.

Histone-histone cross-linking

Cross-linking of histones indicates proximities between them with limits defined by the lengths of the reagent used and the nature of the residues cross-linked, for example, a cross-link formed with dimethyl suberimide indicates that ε-amino groups of two lysine residues (each amino group being about 7 Å from the protein backbone) are within about 12 Å of each other. If two histones can be cross-linked with zero-length reagents they must be in contact. Again, the cross-linking results do not distinguish between the two copies of each histone, but in the model in Fig. 5 this distinction is made.

Five cross-linked histone dimers were originally emphasized (together with histone-DNA cross-linking results) in making the histone assignment in Fig. 5, as follows. With dimethyl suberimide the cross-linked dimers (H3)₂, H3-H4, H2A-H2B, H4-H2A and H4-H2B were found^{6,7}. Of these, (H3)₂ is also formed by mild oxidation²⁸ showing that the single cysteine -SH groups of the two H3 molecules in the chicken erythrocyte nucleosome are close enough to permit oxidation to the disulphide⁵³. H3-H4, H2A-H2B and H4-H2B are cross-linked with zero-length reagents²⁹⁻³¹; and (H3)₂, H3-H4 and H2A-H2B with a short cross-linker (methyl acetimidate)³². The four dimers (H3)₂, H3-H4, H2A-H2B and H4-H2B formed with both long and short reagents of very different specificities and requirements must reflect major histone-histone interactions: these are clearly accommodated in Fig. 5. The pairs H3/H4, H2A/H2B and H4/H2B were also found to interact strongly in solution by physical studies³³. The fifth cross-linked dimer, H4-H2A, produced in roughly the same yield as H4-H2B with dimethyl suberimide⁶, and also found in a later study with a different reagent of similar length³⁴, is also accommodated in Fig. 5.

Figure 5 places the two H3s in contact and represents a strong interaction about the dyad axis as already proposed^{25,28,35,36}. The two H2As are placed on opposite faces and on opposite sides of the wedge-shaped particle; the two H2Bs are also on opposite faces but at the same end of the wedge. H2A and H2B are thus placed as heterodimers, H2A-H2B, consistent with the identical rates of cross-linking of H2A and H2B in chromatin⁶. Our assignment brings H2A and H2B into juxtaposition along, rather than across, the helical ramp, and so accommodates the

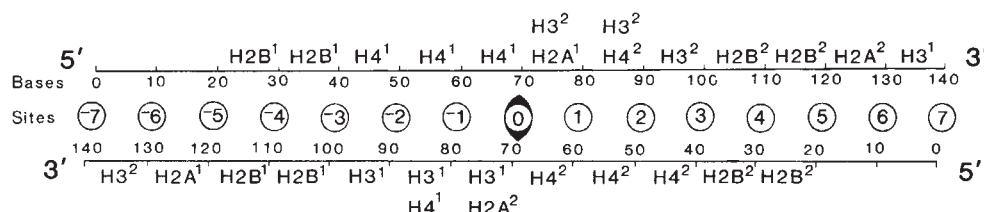


Fig. 6 Assignment of DNA-histone cross-links to individual histone molecules. The diagram is adapted from Fig. 4 of Mirzabekov *et al.*¹⁴ whose results show which histone type can be cross-linked to which location on the DNA, but which cannot distinguish between the two copies of each histone type. Based on the models of the octamer and nucleosome core particles (Figs 4 and 5), we have assigned superscripts 1 and 2 to the pattern of Mirzabekov *et al.*¹⁴ to distinguish the two copies. Note that the action of the overall dyad of the nucleosome is to turn one DNA strand into the other, and to interchange the superscripts on the histones. We have kept the same number in bases along a DNA strand as that used by Mirzabekov *et al.*, although these should be increased by 4% (ref. 66). The diagram incorporates the average stagger of two bases found for the DNase I cutting sites on the opposite strands of the DNA^{67,68}.

finding that on UV irradiation H2A and H2B are linked to each other through a short piece of DNA³⁷. The two H4s, which interact with the two H3s, but weakly, if at all, with each other (see also below), are placed along the sides of the wedge-shaped particle because they interact with both H2A and H2B. An important cross-linked product accounted for by Fig. 5 is the trimer H2A-H2B-H4 generated by successive treatments of chromatin with UV light and tetranitromethane³⁸.

Note that, in the histone arrangement proposed, where the linear order is H2A-H2B-H4-H3-H3-H4-H2B-H2A (Fig. 5), the four major dimers (H3)₂, H3-H4, H4-H2B and H2B-H2A, are all neighbours along the helical ramp. This would be expected from a bifunctional lysine reagent which would have a high probability of cross-linking lysines abundant on a protein surface. As the major function of the lysines of the histone core is to bind the DNA, we would expect them to be particularly abundant in the surface regions where the DNA is bound. The dimers that appear on cross-linking will therefore, as it were, mark out the path of the DNA.

Our assignments accommodate the five major cross-linked dimers referred to above. We now discuss other dimers that have been found (with the longer reagents, 12–15 Å span). Dimers found in reasonable yields are H3-H2B (refs 22, 34, 39, and J. O. T., unpublished results), H3-H2A³⁹, and (H2A)₂ and (H2B)₂²². Although H3-H2A is clearly consistent with Fig. 5, the others are less obviously so. However, the histone shapes are not known, and the H3-H2B dimer can be accommodated if the H2B¹ and H3¹ molecules are elongated to meet each other near the centre of the particle face (see Fig. 5). Likewise H2B molecules of sufficient width could account for an (H2B)₂ dimer; and (H2A)₂ dimers could arise by cross-linking with long reagents across the central cavities.

In addition to the nine cross-linked dimers discussed above, (H4)₂ is also possible in principle, and has been found as 1% of the total dimers generated by formaldehyde⁴⁰. (However, all ten possible dimers are produced with this reagent, and the nature of the cross-linking species is unclear [formaldehyde itself is not bifunctional].) Although (H4)₂ does not seem to represent a major contact, it can be accommodated in the model in Fig. 5 if the H4s are elongated (extended into the 'no-man's land' in Fig. 7b; see below), or it might arise by transient contacts generated as the structure 'breathes'.

Relation of H1 to the other histones in the nucleosome

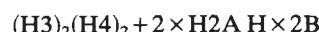
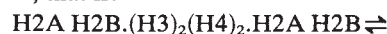
Electron microscopy⁴¹, DNase I digestion⁴² and UV cross-linking³⁷ suggest that in the nucleosome H1 'seals' a two-turn 166-base pair¹⁹ nucleosome core by binding to the DNA where it enters and leaves the nucleosome. Consistent with this, Mirzabekov and his colleagues⁴³ observe strong cross-linking of H1 to 175-base pair DNA over a stretch close to the ends.

By extrapolation from the 146-base pair structure to a complete two-turn particle as in Fig. 5, H1 would bind to the DNA near the (H3)₂ dimer region. This location would explain

the H1-H3 cross-link reported in nuclei and chromatin⁴⁴ (in fact two H3s per H1 are suggested) and also, as H1 is extended, at least in solution^{2,45}, the H1-H2A cross-link found in mononucleosomes (refs 46, 47, and J.O.T., unpublished results). The extended shape of H1 is also invoked⁴³ to explain the cross-linking of H1 at intervals along almost the whole length of the DNA in the 175-base pair nucleosome as well as the major interaction at the ends, but it is not easy to imagine the nature of such binding.

Dissection of the octamer into the (H3)₂(H4)₂ tetramer and H2A-H2B dimers

The histones assigned as above fall naturally into an arginine-rich tetramer and two lysine-rich dimers, and thus accord with the mode of disproportionation of the free octamer in solution^{6,7,9,10}, that is:



The finding of an intermediate hexamer shows that the two H2A-H2B heterodimers can dissociate independently, consistent with their location on opposite faces of our model of the core particle (compare with ref. 48).

If these two heterodimers are removed from the octamer model in Fig. 7a the remaining (H3)₂(H4)₂ tetramer is revealed as a 'distorted horseshoe' (one H4 being above the plane of the other) or, if the H4s meet, as a 'dislocated disk', the uncertainty being reflected in the no-man's land in Fig. 7b. The highly asymmetric shape of the tetramer, if it persists in solution, might explain its anomalously low sedimentation coefficient ($S_{20,w} = 2.81\text{S}$) and resulting high frictional ratio ($f/f_0 = 1.63$)⁴⁹. Others have attributed the slow sedimentation to freely mobile N-terminal regions extending from a more compact globular core⁵⁰, and a contribution from such effects cannot be ruled out.

In the dislocated horseshoe model (Fig. 7b) the two H4 molecules do not touch, and indeed no (H4)₂ is observed on cross-linking the free octamer (J. O. T., unpublished results) or the nucleosome core, except for a few per cent in one study with formaldehyde (see above). However, the (H3)₂(H4)₂ tetramer free in solution, on treatment with dimethyl suberimidate, does give some cross-linked (H4)₂, in about the same amount as (H3)₂ (H3-H4 being the major cross-linked dimer)². This could be due to 'contraction' of the tetramer when the DNA is removed and/or increased possibilities for cross-linking of H4 to itself (and to H3) in the absence of the lysine-rich histones H2A and H2B. As already noted, we do not know where the histone boundaries lie, but probably H4 is elongated since it interacts with the DNA in the nucleosome at three sites about 10 bases apart¹⁴ (see Fig. 6). Whatever the details, the tetramer emerges as a disk-shaped object of approximate thickness 27 Å, consistent with spacings observed in electron micrographs and X-ray patterns of fibres made of H3/H4 histones⁵¹.

The central role of the arginine-rich histone tetramer in the nucleosome

There is now much evidence supporting the proposal of Kornberg⁵ that the arginine-rich tetramer (H3)₂(H4)₂ is critical in forming the nucleosome. As first shown by Felsenfeld and co-workers⁵², and subsequently by others⁵³⁻⁵⁸, H3 and H4, without H2A and H2B, can confer nucleosome-like properties on DNA when assessed by various criteria. These properties were assumed to arise from one tetramer combined with a nucleosome core length of DNA. However, it was subsequently found that H3 and H4 alone can form both tetrameric and octameric complexes with DNA^{59,60} and therefore this interpretation has had to be re-examined. Although it has now been shown⁶¹ that tetramers alone, like octamers, can generate folded complexes about the size of nucleosomes, with long DNA, they do not confer complete resistance of 140 base pairs to nuclease digestion⁶⁰, and the question of just how much a tetramer contributes to the supercoiling of the DNA is not yet settled^{36,53,55,56}.

The structure deduced for the tetramer in Fig. 7b and its location with respect to the DNA (Fig. 5b) explains some of the properties it confers on the DNA in the absence of H2A and H2B. The tetramer spans the diameter of the nucleosome and can be regarded as 'measuring out' the DNA in a turn, agreeing with our earlier suggestion¹⁹. (In the interaction of the tetramer with the central region of the DNA in a nucleosome, the structure resembles 'model 2' of ref. 52 [their Fig. 13].)

Thus a disk-shaped tetramer (Fig. 7b) would be expected to protect the 80 base pairs of DNA in the central turn of the nucleosome from attack by nucleases. This is consistent with the results of Stockley and Thomas⁶⁰ who showed that digestion of long chromatin that had been stripped of all histones except H3 and H4 resulted in two classes of nucleoprotein complexes containing protected DNA; one class contained single H3/H4 tetramers associated with DNA about 70 base pairs long, the other containing an octamer of H3 and H4 and about 140 base pairs of DNA. This explained why micrococcal nuclease limit digests of reconstitutes prepared at an H3/H4:DNA weight ratio of 0.5:1 showed a strong pattern of bands up to about

70–80 base pairs, but only weak or fleeting protection between 80 and 140 base pairs⁵², as at this histone:DNA ratio the nucleoprotein complexes were mainly tetrameric, with only a little octamer⁵⁹. Moreover, the proposed interaction of the tetramer with a turn of DNA would probably mean that a single tetramer alone could induce a degree of supercoiling in relaxed, closed circular DNA, although an octamer of H3 and H4 may be necessary to develop the full number of superhelical turns characteristic of true nucleosomes containing all four histones⁶².

In our model, the tetramer constrains the central 80 base pairs of DNA into one physical superhelical turn, but it can presumably also bind loosely the two additional half-turns in nucleosome-length DNA, because compact tetrameric complexes of nucleosome size and containing about 145 base pairs are formed when (H3)₂(H4)₂ tetramers are reconstituted on to SV40 DNA at low histone:DNA ratios⁶¹, these complexes being less dense than their octameric counterparts. Such folded tetrameric complexes may also be intermediates in reconstitution experiments with 140-base pair DNA, in which 'primed' complexes of H3, H4 and DNA bind H2A and H2B at low ionic strength to regenerate nucleosome cores^{36,48,52,53,63}.

The central role of the (H3)₂(H4)₂ tetramer in nucleosome structure as it emerges from this study is to organize the DNA into the central turn of a 'primary particle'. In nucleosome assembly H2A and H2B could then add to this intermediate as two heterodimers, stabilizing it by binding firmly the two extra half-turns of DNA previously bound loosely in the H3/H4-DNA complex, thereby completing the 166-base pair nucleosome core. H1 would then bind to the core to seal the two turns, and also provide a binding site for an adjacent length of linker DNA. The nucleosome would thus be complete. Such a sequence of events would provide a physical rationale for the temporal order of assembly of histones on to newly replicated DNA^{64,65}.

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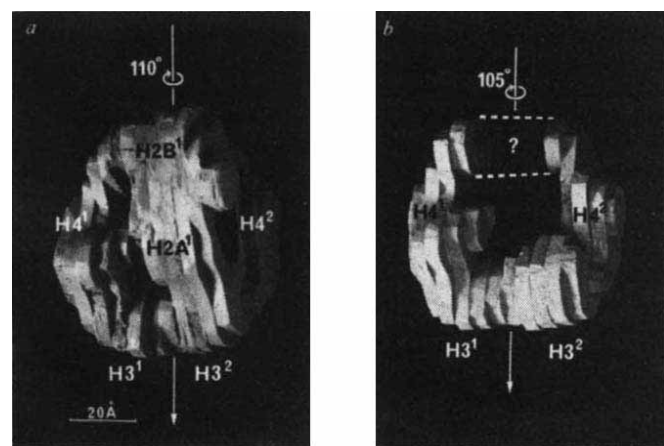


Fig. 7 a, Another view of the histone octamer model rotated about the dyad axis by about 115° with respect to the view in Fig. 3, and hence by 95° to that in Fig. 4a. The thin end of the wedge (in Fig. 3) now runs horizontally at the bottom of the photograph, and appears almost in its full length. The direction of view is similar to that in Fig. 5b. b, The (H3)₂-(H4)₂ tetramer dissected out of the octamer model, the view being about 5° off that in a, to show the shape in its broadest aspect. The boundaries between the tetramer and the two dimers of H2A-H2B removed from the front and back of it have been chosen at the regions of slenderest connection so as to give approximately the appropriate volumes. The region marked ? is a no-man's land which cannot be assigned unambiguously to one or other of the H4 and H2B molecules which border it. The tetramer has the shape of a distorted horseshoe or dislocated disk, so that its periphery constitutes about one turn, or somewhat less, of a flat left-handed helix. The stretch of DNA associated with it runs from about sites -4 to +4. The hole in the middle may be exaggerated by the staining (see text).

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LETTERS

An optical burst from the star identified with the X-ray source 2S1254–690

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High time-resolution photometry of the proposed optical counterpart (Star 30)¹ of the X-ray source² 2S1254–690 is reported here. An event of high statistical significance was observed from the star that has all the temporal characteristics of an X-ray burst (see, for example, ref. 3). 2S1254–690 has not previously been identified as an X-ray burst source, but its optical counterpart shows properties in common with this class of objects. We also derive the X-ray spectrum of 2S1254–690 using data obtained with the HEAO 1 satellite. The spectrum can be fit in the energy range 0.18–30 keV by a power law of photon index –2.5, modified by interstellar absorption.

2S1254–690 Star 30 was observed with the 4-m telescope at CTIO as part of a systematic survey of the photometric properties of X-ray source counterparts. The star was observed in white light with a cooled EMI 9658 R photomultiplier tube with an S 20 photocathode (red extended by prismatic reflection). Data were taken with a 5-arc s diameter aperture and at a time resolution of 10 ms. The observation lasted ~45 min and began at 23.50 UT on 24 June 1979. A total count rate of ~2,400 counts per s (c.p.s.) was recorded on the star, of which ~1,700 c.p.s. were due to the sky background.

At approximately 00.10 UT on 25 June, the total count rate from star plus sky was observed to increase rapidly to ~3,200 c.p.s. on the real time monitor in the 4-m telescope control room. This represents an increase of a factor of two in the stellar count rate. A visual check of the TV guider confirmed that the equipment continued to function normally and the sky conditions were stable. The data collected during this time are plotted in Fig. 1 accumulated in intervals of 0.2 s. The event observed is remarkably similar to the X-ray bursts identified in several galactic sources. It has a rise-time of ~1.7 s and then decays more slowly, having a total duration of ~20 s. The data are consistent with a morphology that has either a relatively flat maximum, or alternatively a second peak in flux that occurs

about 5 s after onset. Again, such a multiply peaked, or flat-topped, structure has been observed in X-ray bursts³. No other event of this kind was observed during the remainder of the observation of 2S1254–690.

Because of the marked similarity in structure between the optical burst we have detected from 2S1254–690 Star 30 and X-ray bursts, we believe that we probably observed the optical counterpart of such an X-ray burst from 2S1254–690. Optical bursts of lesser statistical quality have been reported from three burst sources, MXB1735–44 (ref. 4), MXB1837+05 (ref. 5) and MXB1636–53 (ref. 6) coincident with X-ray bursts. The observation of burst-like behaviour from 2S1254–690 Star 30 is consistent with its identification as the optical counterpart of the X-ray source. No X-ray bursts have been reported from 2S1254–690 thus far, and this means that it is the first bursting source to be catalogued in the optical region of the spectrum. However, we note that 2S1254–690 does lie within the 12° radius error circle of an X-ray burst observed by Apparao with the SAS 3 rotation modulation collimator, whose position was reported by Lewin and Clark³.

We have searched for evidence of periodic modulation in the light from 2S1254–690 Star 30 by constructing Fourier transforms of the data. No spike due to periodic modulation above the 1.2% pulsed fraction level was observed from 0.02 to 50 Hz in a power spectrum constructed from the entire data-set.

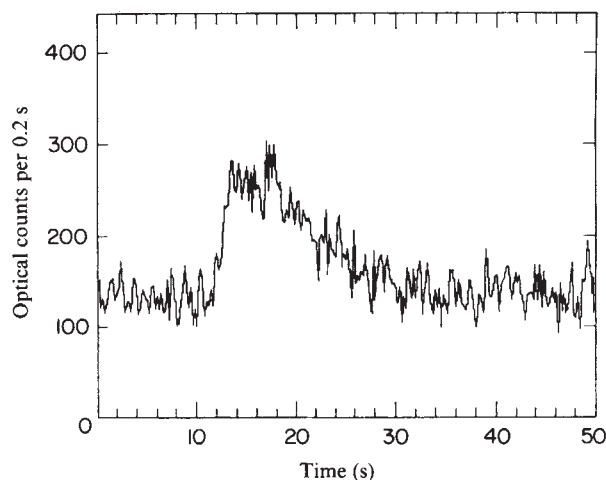


Fig. 1 Time series obtained in white light on the CTIO 4-m telescope in the vicinity of the burst-like event observed from 2S1254–690 Star 30. Data are accumulated in 0.2-s intervals and time is measured on the abscissa in seconds from 25 June 00.08.43 UT. The ordinate shows the excess count per 0.2-s bin above sky background registered on the star.

Table 1 HEAO 1 0.18–20 keV spectral fits for 2S1254–690

Spectral model	α/kT	$\log(N_H)$	Line			χ^2 min	D.f.
			Energy	EW	FWHM		
Power law	2.38	21.44	—	—	—	132	71
Exponential + gaunt factor	—	—	—	—	—	363	71
Black body	—	—	—	—	—	~1,000	71
Power law + narrow line	2.46	21.48	6.4	0.4	—	99	69
Power law + broad line	2.47	21.51	6.5	0.6	1.6	92	68

However, when data only from the immediate vicinity of the burst are considered, a peak is found at a frequency of 36.40 Hz that is 12.6 times the average power at neighbouring frequencies. The *a priori* probability that any bin in our 4,000-point transform would have this much power as a result of a random statistical fluctuation is 1%, so that the peak at 36.40 Hz is statistically an unusual event. The periodicity is present in the 20 s of data taken during the burst and also in a data-set that includes the 7-s interval preceding the burst and the 13-s interval following it. The mean pulsed fraction is ~5% of the mean count-rate from the star.

The pulsation is too weak to enable us to determine its properties more precisely although if the effect is confirmed, the implied size of the optical emission is only ≤ 0.3 light s. Experimenters using the A1 experiment on the HEAO 1 satellite have reported an 83-Hz periodicity in one X-ray burst from MXB1728–337 (Sadeh and Wood, personal communication). The period of the pulsation, which was detected both before and during the burst, changed by a significant amount during the time it was observed, and was not seen in three other bursts observed from the same source. High time-resolution observations of bursts to investigate the nature of these pulsations clearly deserve more attention.

The known optical characteristics of 2S1254–690 Star 30 are summarized elsewhere¹. The star ($V=19.1$) has a UV excess, and may exhibit line emission at λ 4,640 Å corresponding to the C III–N III blend often seen in X-ray sources. These properties are similar to those of several galactic X-ray source counterparts, including several bursters.

We have investigated the X-ray properties of 2S1254–690 using scanning data taken with the proportional counters of the A2 experiment⁷ on HEAO 1 in August 1977, February 1978 and August 1978. The source was visible for periods of 2.5, 5.0 and 5.0 days respectively on these occasions. The overall flux of 2S1254–690 was constant throughout to within ~15% on time scales of ~1 h. The sensitivity of the experiment to short time-scale bursts is, however, low because the source is within the largest field of view of the instrument (6° FWHM) for at most one minute every ~30-min satellite rotation period.

To investigate the X-ray spectral properties of 2S1254–690, we have examined the pulse height analyser data taken with the low, medium and high energy detectors (LED, MED and HED respectively) of the A2 experiment. These data have been modelled in turn with power-law, exponential plus gaunt factor and black-body models, allowing for absorption in the interstellar medium in each case using the elemental absorption cross-sections given by Brown and Gould⁸.

The results of fitting these models to the combined data from the LED (0.18–2.5 keV) and MED (2.0–20 keV) detectors, which together provide data in 75 energy channels, are summarized in Table 1. The relative normalization of the spectrum in the two detectors is a free parameter in the fits in order to allow for small uncertainties in their relative effective area. Of the three simple models tried, only a power law gave a reasonable fit to the data (minimum χ^2 of 132 for 71 d.f.). This is true also when the MED and HED (2–60 keV) data are analysed separately. The quality of the power law fit was further improved by the addition of an emission line at ~6.5 keV. For the combined LED and MED data, a model that included a narrow

line resulted in a minimum χ^2 of 99 for 69 d.f., while the best fit for a power law plus a broad line gave a minimum χ^2 of 92 for 68 d.f. The line had a best-fit energy of 6.5 ± 0.5 keV, an equivalent width of 0.6 ± 0.1 keV, and a FWHM of 1.6 ± 0.5 keV. It may be fluorescent iron line emission similar to that found in some pulsating X-ray sources (see for example ref. 9). The allowed range of power law slope is expressed in Fig. 2 as a function of low energy absorption column density. Figure 2 also shows the best-fit spectral model, which has a photon spectral index of $\alpha = -2.47$ (energy index -1.47) and a column density, N_H , of 3.2×10^{21} equivalent H atoms cm^{-2} .

The HEAO 1 spectral measurements of 2S1254–690 are consistent with the work of Jones¹⁰ on this source. The spectrum is similar in overall slope to the X-ray spectra of most burst sources that have been studied¹¹ (an exception is the burster 2S1916–053 which has a hard spectrum¹²). The form of the spectrum, however, a power law, is notable. The HEAO 1 data show that a power law representation of the spectrum of 2S1254–690 is clearly preferred over simple exponential or black-body models in the energy range 0.18–20 keV (Table 1). Furthermore, data from the 2–60-keV HED detectors of the A2 experiment confirm that the power law measured from the LED

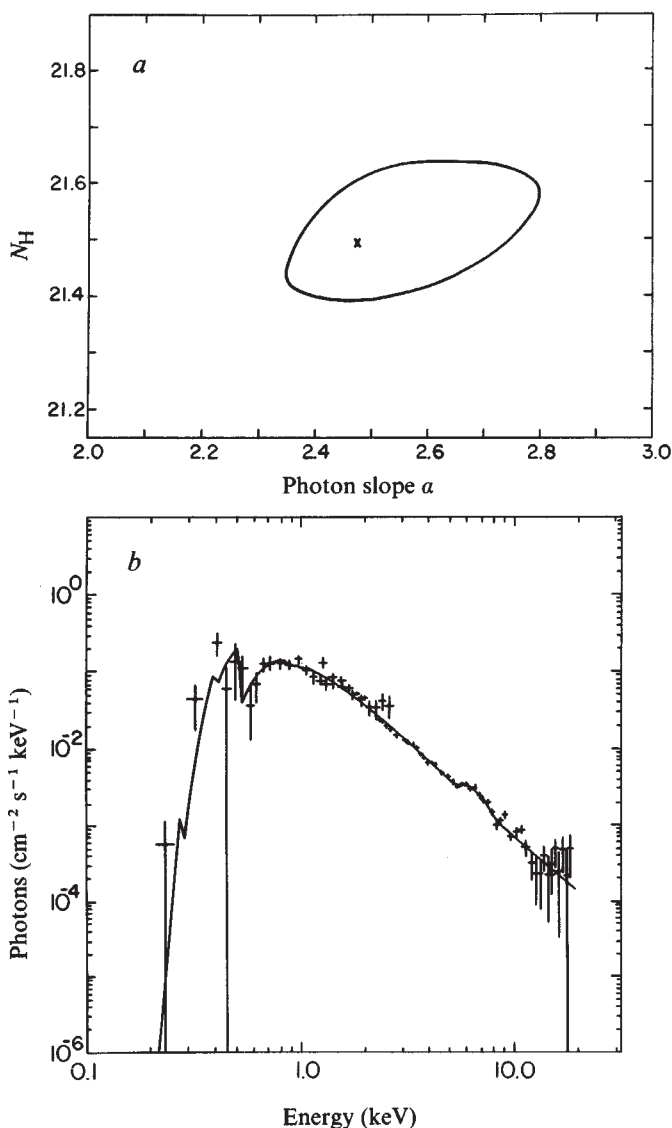


Fig. 2 The 0.18–20-keV spectrum of 2S1254–690 derived from data obtained with the HEAO 1 A2 experiment. *a*, Allowed range of photon index and absorption column density for a power law model plus a broad line. The solid line encloses the 90% confidence region. *b*, Best-fit photon spectrum shown relative to the corrected MED and LED data.

and MED data adequately describes the spectrum of 2S1254–690 up to an energy of at least 30 keV. In general, the incidence of power law spectra among the 'population II' X-ray sources that have been studied to date is low (see, for example, refs 13, 14). One X-ray source whose 2–12-keV spectrum sometimes has a power law distribution is 4U0614+09, which is also suspected of being a burster¹⁵. 4U0614+09 was observed on two occasions by the collimated proportional counter¹⁶ on Ariel V. During the first observation which lasted ~2 days the source was relatively steady, varying by less than 10% on a time scale of ~100 min. The integrated 2–12-keV count spectrum from this observation has been modelled by one of us (K.O.M., unpublished). A power law spectrum, although not statistically acceptable (due in part to systematic uncertainties in the detector response at low energies and in part to a probable excess near ~6.5 keV), yields a minimum χ^2 value which is a factor of three below that obtained by fitting an exponential plus gaunt factor shape. The best-fit power law photon index was $\alpha = -2.6$, close to that found for 2S1254–690. During the second Ariel V observation¹⁶ of 4U0614+09, the source was more active and showed variations in intensity by up to a factor of two. The OSO 8 satellite detected a burst from the vicinity of 4U0614+09 during the same period¹⁵. The variation in the strength and spectrum of the source precluded addition of data from more than one orbit, but on an orbit by orbit basis, exponential + gaunt factor spectra provided consistently better fits to the Ariel V data than a power law, in contrast to the first observation (see also ref. 14).

Thus we have evidence that at least one optical burst source, 2S1254–690, and a suspected X-ray burst source, 4U0614+09, occasionally emit power law X-ray spectra. Power law X-ray spectra are usually associated with non-thermal mechanisms (for instance, synchrotron radiation) or inverse compton radiation from a hot relativistic accretion disk¹⁷. If X-ray bursters are accreting neutron stars, this goes against naive expectations that X-ray emission from such stars should be essentially thermal in shape^{18–20}. However, it is also possible that a suitable combination of thermal spectra of different temperatures might simulate a power law distribution over a limited energy range. The detailed observation and classification of X-ray source spectra could be a useful way to investigate the nature of these objects, but a better theoretical understanding of emission from compact stars²¹ is probably required before such studies can be used to constrain the physical processes occurring there.

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The 3.4- μ m interstellar absorption feature

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Estimation of the organic component of interstellar grains has provoked considerable interest^{1,2}. Recently Duley and Williams² concluded from an absence of the 3.4- μ m CH absorption band in obscured IR sources that organic compounds in grains account for $\leq 10\%$ of carbon in the interstellar medium. We report here the detection of a 3.4- μ m absorption feature in three IR sources and hence argue that an appreciable mass fraction of interstellar grains are predominantly organic in character.

The presence of a possible absorption feature in the wavelength region 3.3–3.5 μ m in the published spectra of some highly obscured IR sources was considered by Hoyle and Wickramasinghe³ to be evidence for the ~3.4- μ m stretching vibration band of CH, which is characteristic of most organic molecules. In most cases for which data have been available the 3.4- μ m feature is seen in the wing of a broad, strong water-ice band centred at 3.1 μ m. Although the difficulty of matching the extended red wing of the 3.1- μ m feature by water ice alone has been noted⁴, the presence of CH absorption has not been established. In deriving their limit, Duley and Williams² used an uncertain upper limit of optical depth $\tau(3.4) \leq 0.05$ for a visual extinction $A_V = 70$ mag which they estimated to be consistent with current astronomical data. We now discuss observations of five IR sources having $10 < A_V < 35$ which lack the 3.1- μ m ice absorption band and determine or place strong limits on the strength of the 3.4 μ m CH absorption feature.

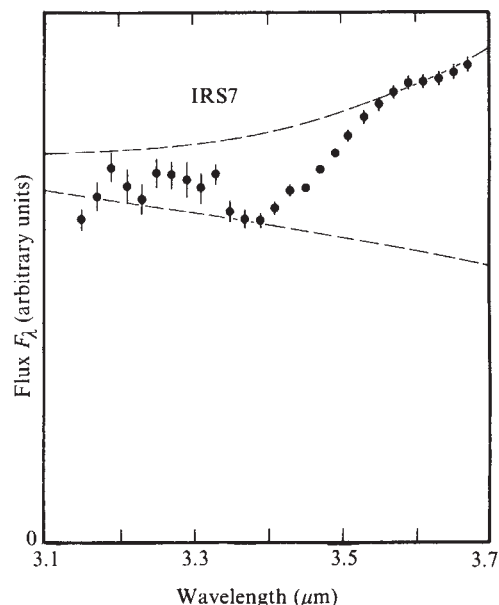


Fig. 1 Spectrum of the galactic centre source IRS7 showing the 3.4- μ m absorption band. The upper and lower curves are discussed in the text.

The observations were made in February and April 1980 with the 3.9-m Anglo-Australian Telescope and the IR photometer-spectrometer. The IR sources, all optically invisible, include two in the galactic centre region⁵, two in the Coalsack⁶, and one recently discovered by Jones and Caswell (personal communication) coincident with an OH source at the 1950 coordinates 17 h 46 min 12.5 s, $-27^{\circ} 41' 03''$. For all sources we acquired photometry at 1.25, 1.65 and 2.2 μm , and spectra with $\lambda/\Delta\lambda \sim 50$ in the wavelength regions 2.0–2.5 and 3.1–3.7 μm . Stellar spectral types were derived from our shorter wavelength spectra, and these were combined with the photometry to derive values of A_V , using the interstellar extinction data of Jones and Hyland⁷. The latter data partly parallel other published values, and the agreement is good except that for the galactic centre sources we find somewhat earlier spectral types (and hence larger extinction) than Neugebauer *et al.*⁸. The longer wavelength spectra were searched for absorption near 3.4 μm . Table 1 details the results. The 3.1–3.7 μm spectrum of IRS 11 has a low signal-to-noise ratio. The limit $\tau(3.4)/A_V \leq 0.004$ for the Ophiuchus dark cloud⁹ may be added to the data of Table 1.

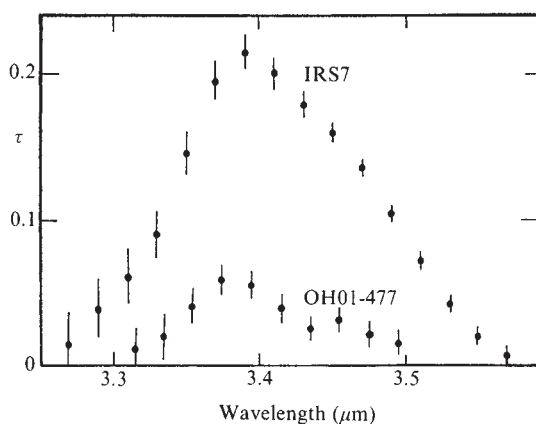


Fig. 2 Band profiles in IRS7 and OH01-477. The optical depth τ is calculated as described in text.

Figure 1 shows the 3.4- μm absorption feature in the source IRS7. The lower continuum in Fig. 1 is the expected flux for the M4 supergiant extrapolated from our 2- μm data with the Jones and Hyland reddening law assuming $A_V = 34$ mag. The observed excess is clearly due to circumstellar dust. We estimate the excess in this wavelength region to correspond to a reddened 300 K black body on the basis of the spectrophotometry data of Willner *et al.*¹¹. The resulting continuum fitted at 3.6 μm is shown as the upper curve in Fig. 1 and demonstrates that the observed feature is an absorption at 3.4 μm . The residual structure in the first five points of Fig. 1 probably reflects the failure to correct wholly for the strongest of the telluric water vapour bands at 3.2 μm . The presence of a possible absorption feature near 3.4 μm in the spectrum of IRS7 has already been reported^{10,11}. Our data provide unambiguous evidence for the existence of this band and define its characteristics.

Figure 2 is a plot of the band shape smoothed to a resolution of 0.08 μm . In deriving the optical depth we have allowed the continuum at the short wavelength region to be lowered to fit our data at 3.2–3.3 μm . This may have artificially steepened the short wavelength edge of the band but the central wavelength is not affected. We find the wavelength of the peak absorption to be 3.99 ± 0.02 μm , the equivalent width to be 330 Å and the intrinsic FWHM to be 0.13 μm . A similar procedure was adopted with OH01-477. The band shape included in Fig. 2 is the weighted mean of two observations. We have not attempted to deduce a band shape for IRS11 because the detection of the 3.4- μm feature in this source is at the 3 σ level.

Table 1 Derived values of A_V and $\tau(3.4)$ for the programme sources

Source	Spectral type	A_V	$\tau(3.4)$	$\tau(3.4)/A_V$
Coalsack 3-1	M6	11	≤ 0.06	≤ 0.005
Coalsack 2-2	M0	15	≤ 0.06	≤ 0.004
OH01-477	M4	19	0.06	0.003 ± 0.001
GCIRS11	M4	30	0.2	0.006
GCIRS7	M4I	34	0.22	0.006 ± 0.001

We believe that the 3.4- μm absorption arises in the general interstellar medium. The main arguments against a circumstellar origin are: (1) The feature is present in the three most highly obscured sources. (2) We find no evidence of 3.4- μm absorption in the M supergiant VX Sgr, nor in M giant stars. Moreover we expect none in oxygen-rich stars such as those we consider here where carbon preferentially forms CO. (3) Becklin *et al.*¹² argued from the similar colours of the bluest galactic centre sources that most of the reddening towards IRS7 is interstellar.

We discount an origin in the dense molecular clouds, where absorption due to other CH and CC bonds is known to arise¹³ because: (1) sources known to be obscured by molecular clouds show absorption at 3.1 μm ; and (2) The line of sight towards IRS 7 and 11 does not pass through the molecular clouds mapped by Solomon *et al.*¹⁴. Finally the width of the 3.4 μm absorption feature argues against an origin in the gaseous phase in low temperature diffuse interstellar clouds.

Using laboratory data on the strength of the 3.4- μm CH band in various common organic molecules, Duley and Williams² have related the optical depth $\tau(3.3-3.4$ $\mu\text{m})$ at peak absorption to the total visual extinction A_V assuming that all the carbon is in the form of a particular organic species. We may now use these results and our observations to estimate the fraction of carbon that is required to be organic material. We find that $\sim 10\%$ may exist in alkanes, whereas the unsaturated hydrocarbons (alkenes, alkynes or aromatics) would account for the visual extinction as well as the 3.4- μm absorption with no requirement for any other grain material.

The difficulty of fitting the extinction curve at all observed wavelengths using a distribution of grains of one specific composition is well known. A grain model based entirely on organic molecules is also clearly ruled out on the basis of the high depletions observed for gas phase metals in the interstellar medium. The present observations do, however, suggest that an appreciable fraction of the observed IR and visual extinction may arise from grains that are largely organic in character.

We thank Bob Carswell for permission to take some of the daytime observations, and Harry Hyland and Terry Jones for drawing to our attention OH01-477. D.T.W. thanks Professor Chandra Wickramasinghe for pointing out the significance of establishing the presence of a 3.4- μm interstellar extinction feature, and Professors F. Hoyle, P. M. Solomon and K. Nandy for valuable discussions.

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Lunar asymmetry and palaeomagnetism

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The compositional asymmetry between the nearside and farside of the Moon and the natural remanent magnetism (NRM) of lunar rocks are poorly understood. The compositional asymmetry is indicated by the 2-km offset towards the Earth of the centre of mass relative to the centre of figure and the concentration of both KREEP and mare basalts on the nearside. Wasson and Warren¹ recently noted that these asymmetries may be better explained by an asymmetric crystallization of a primordial magma ocean than by the often proposed²⁻⁴ greater thickness of farside anorthositic crust. The NRM⁵ has been attributed to an ancient lunar dynamo^{6,7}. I propose here a model for the early lunar evolution in which the preferred gravitational energy state consisted of an asymmetric accumulation of a liquid iron alloy (Fe-Ni and a small amount of sulphur) which displaces upwards the cold, primordial, undifferentiated core. The resulting depth asymmetry of the outer partially molten zone leads eventually to the subcrustal accumulation of light, magnesium-rich pyroxenes and olivine, preferentially in one hemisphere, sufficient to explain the offset and also indirectly providing a possible explanation for the nearside concentration of KREEP and mare basalt. Meanwhile, slow downward migration of the iron releases gravitational energy sufficient for convection and dynamo generation in an iron layer for about 10^9 yr. The proposed present state of the Moon has a symmetrically placed iron core (radius ≈ 500 km), unlike a previous model for the lunar asymmetry⁸.

A crucial assumption in the model is an early Moon consisting of a cold interior region and a hot outer region, consistent with (but not limited to) conventional accretion models^{9,10}. The outermost few hundred kilometres are frequently modelled as fully molten (the putative magma ocean) but this is neither geochemically essential nor geophysically plausible. A balance of likely accretional energy input with the outward heat flow suggested by Nusselt-Rayleigh number convection recipes¹¹ suggests that this region will self-regulate to a viscosity within a few orders of magnitude of 10^{16} P, for which substantial partial melting (essential to explain the anorthosite crust and KREEP) may be needed¹². For such a viscosity, the convective heat transport is then ~ 100 -fold greater than the present heat output, sufficient to eliminate the excess accretional energy input in about 10^8 yr. Boundary layer theory¹³ then predicts a convective overturn time $\sim 10^5$ yr, sufficiently smaller than the evolution time scale to ensure lateral homogenization.

The absence of siderophiles in lunar rocks and the low oxygen fugacity of lunar magmas enable metallic Fe-Ni (ref. 14) to be present, although the lunar density, moment of inertia and magnetic measurements¹⁵ limit the amount to $\leq 5\%$ by mass. Iron droplets (maintained at least partially liquid by a small amount of sulphur) can readily precipitate to form a thin layer above the cold, undifferentiated primordial core. However, this situation is spontaneously unstable because a lower gravitational energy U can be achieved if the iron accumulates on one side, forming a crescent-shaped region and displacing the much less dense primordial core upwards (Fig. 1). For a displacement h , the change in U is approximately $\Delta\rho_1 g \pi R_c^2 h^2 - \Delta\rho_2 g V_{Fe} h$, where $\Delta\rho_1 \equiv \rho_c - \rho_m$, $\Delta\rho_2 \equiv \rho_{Fe} - \rho_m$, ρ_c is the primordial core density, ρ_m is the (partially molten) mantle density, ρ_{Fe} is the liquid iron alloy density, g is the gravitational acceleration, R_c is the primordial core radius and V_{Fe} is the volume of precipitated

iron. As compressional effects are negligible, the preferred h is given by minimizing U and is therefore approximately $1/2(\Delta\rho_2/\Delta\rho_1)V_{Fe}/\pi R_c^2$. More detailed, rigorous calculations change this approximate result by $\leq 30\%$.

For $\rho_c = 3.34 \text{ g cm}^{-3}$, $\rho_m = 3.15 \text{ g cm}^{-3}$ (corresponding to 25% partial melt, an arbitrary choice), $\rho_{Fe} = 7.0 \text{ g cm}^{-3}$, $R_c = 1,300 \text{ km}$ and $V_{Fe} = 3 \times 10^{23} \text{ cm}^3$ (that is, 5% iron by mass), the predicted value of h ($\sim 600 \text{ km}$) exceeds the depth of the mantle. This means that the primordial core could be displaced to the lunar surface. If a true magma ocean were possible ($\rho_m = 2.8 \text{ g cm}^{-3}$), h would be $\approx 200 \text{ km}$, still a large effect. This asymmetry would have no lasting consequence if the iron could migrate to the centre in $\leq 10^8$ yr. However, core formation in the Moon was much less rapid than in the other terrestrial bodies because of the low gravity (implying low deviatoric stresses associated with the asymmetry) and the low free iron abundance. The sinking layer¹⁶ and Rayleigh-Taylor (Elsasser drop-plet¹⁷) core formation modes are slow ($\sim 10^9$ yr) because the primordial core is cold and downward transport of heat is inefficient¹⁸. Stresses associated with the asymmetry are 0.1–1 kbar (limited in part by the low stress level for an $l=1$ spherical harmonic of the load¹⁹) but the resulting cold deformation will not eliminate the asymmetry.

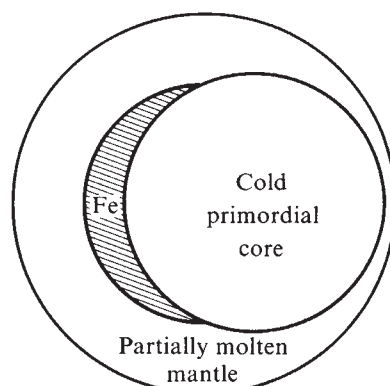


Fig. 1 Schematic representation of the energetically preferred asymmetric arrangement of matter within the early Moon, after precipitation of a liquid iron alloy from the partially molten mantle. The magnitude of the asymmetry is not well known but could be large (see text). The right-hand side is identified with the present nearside of the Moon (but the early orientation is irrelevant because the Moon's orbit has already tidally evolved to a large Earth-Moon separation).

As the asymmetry develops, partially molten mantle will flow into the deep region above the iron layer. Because the mantle adiabat is much less steep than the liquidus or solidus²⁰, this material will undergo some pressure-induced freezing. Subsolvus, high-viscosity material tends to be stagnant (an analogous situation is observed in convection experiments on fluids with strongly temperature-dependent viscosity²¹) and there will be a tendency for the least fusible solid fraction to accumulate preferentially in the deepest mantle regions. These are the magnesium-rich pyroxenes and olivine²⁰ (less dense than the average Moon by $\sim 2\%$, but more dense than the early, partially molten mantle). Subsequently, at about 4,200 Myr BP, when the degree of partial melt in the outer regions has decreased (because of gradual cooling as surface bombardment decreases) and the anorthositic crust has formed, these light crystallites will rise to beneath the farside crust. A layer of thickness $d \leq 200 \text{ km}$ on the farside, thinning to zero thickness beneath the nearside crust, gives an offset between centre of mass and centre of figure of about $1/2(\Delta\rho/\rho)d$, about the right amount for a density contrast $\Delta\rho \approx 0.06 \text{ g cm}^{-3}$ between the light crystallites and the rest of the upper mantle. The characteristic time scale for lateral

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spreading of this layer is about $3\mu R^2/\Delta\rho g d^3$ (a generalization of ref. 22), where R is the radius, and this time scale exceeds 5×10^9 yr provided the viscosity $\mu \geq 10^{22}$ P. This constraint should be satisfied as the material has a high solidus temperature and is within the thermal boundary layer, even that of 4×10^9 yr ago¹¹. The low stress level for an $l = 1$ spherical harmonic of the load also helps prevent efficient lateral spreading¹⁹. The upwelling of crystallites may also sweep the last dregs of partial melt (from which KREEP was presumably derived²³) around towards the nearside, thereby perhaps explaining the asymmetric KREEP distribution. The nearside predominance of mare basalts later in the evolution can be attributed to the presence of primordial, more fusible material and greater radiogenic heating in the nearside mantle¹.

Meanwhile, silicate grains detach from the deep side of the primordial core and float upward through the iron layer. The detachment rate is determined by the slow downward heat transport¹⁸ (the time scale of iron core formation T_c may be as long as 10^9 yr) and the total gravitational energy released is about 1×10^{35} erg (ref. 24). Although insignificant if averaged over the whole Moon, this energy release is important for the iron layer. For an adiabatic temperature gradient of 2×10^{-6} K cm⁻¹ and a thermal conductivity of 5×10^6 K⁻¹ cm⁻¹, the integrated conductive heat transport along a liquid iron adiabat is 5×10^{25} T_c erg (T_c in yr), less than the energy released provided $T_c \leq 2 \times 10^9$ yr. Superadiabaticity and convection must ensue in the iron layer, and an application of mixing length theory²⁵ suggests a convective velocity $v \sim 0.1$ cm s⁻¹. The corresponding magnetic Reynolds number $R_M \equiv vd/\lambda \sim 10^2$ for a layer depth $d \sim 10^7$ cm and the magnetic diffusivity $\lambda \sim 2 \times 10^6$ cm² s⁻¹ appropriate to liquid iron. Even for the slow rotation of the Moon (angular velocity $\Omega \sim 2 \times 10^{-6}$ s⁻¹), $v \ll \Omega d$, so the Coriolis force is dynamically important and dynamo generation should be possible²⁶. The integrated ohmic dissipation is about $10^{23} H^2 T_c$ erg for a characteristic current $\sim H/d$, and as this is of the order of 10^{34} – 10^{35} erg, it follows that $H \sim 10$ G (the Carnot efficiency factor being irrelevant for gravitational stirring²⁷). This may be the toroidal field, but it is energetically possible to have an external field comparable to that inferred from palaeofield studies, without invoking exotic energy sources such as superheavy elements²⁸.

Because g and the rate of gravitational energy release decrease as the iron approaches the centre, the field H decreases with time and dynamo generation will cease (for lack of an energy source sufficient to drive convection) once a central iron core forms, at $\sim 3,000$ Myr BP. However, several problems remain in relating lunar magnetism to an ancient lunar dynamo²⁹, and the model presented here does not attempt to resolve them all. More important than the details of the model are the basic ideas: an inevitable asymmetry and the likelihood of an ancient lunar dynamo. The model is testable (in principle) by lunar seismic studies designed to detect nearside–farside mantle variations and the existence (still hypothetical) of an iron core.

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Radar meteor rates and atmospheric density changes

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As a way of explaining the evidence linking recorded radar meteor rates with solar activity, it has been suggested that variations of the atmospheric scale height at meteor ablation altitudes, themselves controlled by the Sun, may be responsible. A quantitative examination of a theoretical treatment by Kaiser (personal communication) now shows that the above suggestion is certainly plausible, and details the necessary scale height changes as a function of the meteor mass exponent.

Using observations made in New Zealand, Canada and Sweden, Lindblad¹ and C.D.E.^{2,3} have provided evidence suggesting a link between the apparent radar meteor echo rate and the solar cycle. The phase of this correlation is such that the apparent radar meteor rate increases towards the time of solar minimum. Lindblad has also claimed a connection relevant to short-term periodicities⁴. This variation in meteor rate seems to be an atmospheric effect, and the explanation proposed⁵ is a Sun-controlled variation of the atmospheric density gradient at meteor ablation heights (80–120 km), probably modulated by solar X-ray flux. However, an anomalous increase in the meteor rate was recorded around 1963, greater than that ascribed to the solar effect^{6,7}; this has been ascribed⁵ to an abrupt decrease in solar X-ray output. We, however, consider that this anomaly shows a much higher correlation, both spatially and temporally, with an increase of particulate matter in the atmosphere⁸. Nevertheless, we believe that a change in atmospheric scale height must be responsible for the observed change in echo rate.

A decrease of atmospheric scale height (that is, an increase of density gradient) will bring about this ablation of a meteoroid of a given size over a shorter path length. (Correlation with path length has in fact been noted⁵.) It might thus be reasonably expected that, because the total energy release is (relatively) independent of path length, the shorter path length would be brighter, with a concomitant increase of linear electron density.

With regard to radar observations, however, an increase of linear electron density is offset by the shorter path length, although Lindblad¹ (who was monitoring both trail length and echo amplitude) has not observed this to be the case. The absence of a quantitative treatment of this problem has made some workers sceptical of the suggested link between scale height and observed rate. Baldwin and Kaiser⁹ have suggested that variability in D-region ionospheric absorption may be a more important factor. However, our quantitative study shows that, at least for larger values of the mass exponent s , the large variation in observed meteor rate that C.D.E. requires³ can be accounted for by appropriate changes of density and scale height.

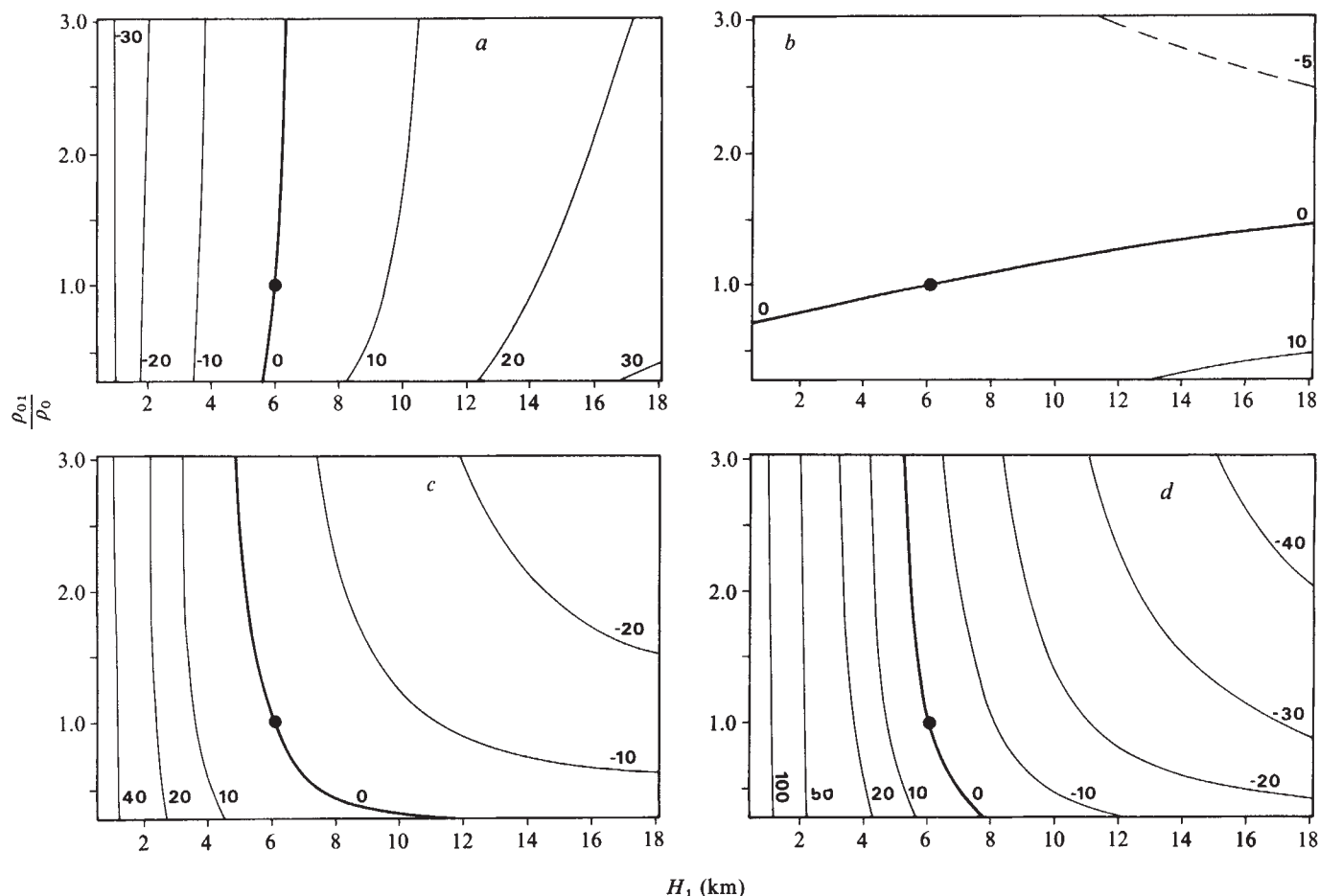


Fig. 1 Percentage change in observed meteor echo rate as a function of atmospheric parameters and meteor mass exponent s where $s = 1.8(a)$, $2.0(b)$, $2.2(c)$ and $2.4(d)$. Change is expressed as $100(N_1 - N)/N$ where N is the observed rate at a scale height of 6 km and density ρ_0 at height 100 km, and N_1 is observed rate for a scale height H_1 and density ρ_{01} .

Using classical ablation theory for compact meteoroids¹⁰⁻¹⁴, Kaiser has shown that

$$\frac{N_1}{N} = \left[1 + \left(\frac{5-3s}{2} \right) \left(\frac{H_1}{\bar{h}} \ln \frac{\rho_{01}}{\rho_0} - \frac{H_1}{3\bar{h}} \ln \frac{H_1}{H} \right) \right] \left(\frac{H_1}{H} \right)^{2-s}$$

where N is the observed rate at a density scale height of H , $\bar{h}$ is the mean height (determined by atmospheric parameters) around which the meteor reflection points are distributed, ρ_0 is the atmospheric density at $\bar{h}$ for the rate N , N_1 is the observed rate for a new density scale height of H_1 and a density ρ_{01} at $\bar{h}$, and s is the exponent in the meteor mass distribution curve, which for sporadic meteors has been experimentally determined to lie between about 2.0 and 2.3. (Simek and McIntosh¹⁵ have obtained a value of $s = 2.35 \pm 0.1$ for the radar sporadic background in the magnitude range of interest.)

A computer analysis of this equation has been carried out to ascertain the percentage change in observed meteor rate as a function of the three variables ρ_{01} , ρ_0 , H_1 and s . These results are presented graphically in Fig. 1 for values of s between 1.8 and 2.4. The values for $\bar{h}$ and H have been taken to be 100 km and 6 km, respectively.

It is immediately evident from Fig. 1 that the percentage change of observed meteor radar rate is sensitive to s . For $s = 2.0$, no combination of density scale height and absolute density change will produce much more than a 5% change of meteor rate. As might be expected from an inspection of the equation, $s = 2.0$ represents the point about which the graphical contours exhibit a marked change of form. For $s < 2.0$ a decrease of scale height actually results in a decrease of meteor rates, contrary to simple expectation. However, as s increases

above 2, the magnitude of the rate increase with decreasing scale height quickly grows.

The maximum rate increase to be accounted for, excluding the abnormal 1963 peak, is about 24%, and for $s \geq 2.2$, such an increase is possible. A halving of scale height from 6 km to 3 km will produce this percentage increase with $s = 2.3$. At this reduced scale height, which corresponds, of course, to a doubling of the air density gradient, the rate is relatively independent of the absolute air density. For $s = 2.5$, only a 30% decrease in scale height is necessary to produce the required increase of meteor rate.

In conclusion, it is possible for change of atmospheric density gradient at meteor ablation heights to have produced the changes noted in the observed radar meteor rate. Further and continuing measurements of the relevant atmospheric parameters in the meteor region are desirable in order to throw further light on this problem.

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A laboratory study on the formation of nitrous oxide by the reaction $\text{N}_2(\text{A}^3\Sigma_u^+) + \text{O}_2 \rightarrow \text{N}_2\text{O} + \text{O}$

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Metastable $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules are produced copiously in auroral substorms by the impact of low-energy secondary electrons on N_2 and by the absorption of cosmic rays and solar X-rays at *E*-region altitudes and below. The major loss process for $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules below 100 km is collisional deactivation by O_2 . We present here the results of a detailed study of the reaction $\text{N}_2(\text{A}^3\Sigma_u^+; v' = 0, 1, 2) + \text{O}_2 \rightarrow \text{N}_2\text{O} + \text{O}$, and we show that N_2O is formed with an effective yield of 0.6 with a probable absolute error of $\pm 30\%$.

Harteck and Dondes¹ have shown that the irradiation of air by high-energy neutrons produces N_2O quite efficiently with an energy cost of 33 eV/ N_2O molecule. Cylindrical shock wave studies simulating lightning have produced similar results². However, neither of these investigations sheds light on the details of the reaction(s) leading to the synthesis of nitrous oxide. In a recent study in which $\text{N}_2\text{--O}_2$ mixtures were bombarded by 14 MeV neutrons, only two products, N^{13}N and N^{13}NO , were observed³. Malcombe-Lawes³ suggested that the reaction



was the principal source of the observed nitrous oxide.

We have carried out a detailed study of this process, and we find that reaction (1) forms N_2O with a quantum yield of $0.6 \pm 30\%$. As metastable $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules are formed efficiently by electron impact with a total cross-section which is quite large⁴ ($\sim 1.4 \times 10^{-16} \text{ cm}^2$ at 15 eV), these energy-rich molecules (and in turn N_2O) will be produced copiously by the low-energy electrons created by cosmic rays in the mesosphere and stratosphere, by solar X-ray and EUV absorption in the *D* and *E* regions and by the secondary electrons produced by high-energy particles precipitating into the auroral zone.

A block diagram of the plasma spectroscopy apparatus and a detailed description of the techniques used in this study are given elsewhere⁵. Only a brief summary of these techniques will be presented here. $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules were formed inside a cylindrical stainless steel cavity by a periodically pulsed microwave discharge. The behaviour of the $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules in the discharge and afterglow period was monitored by

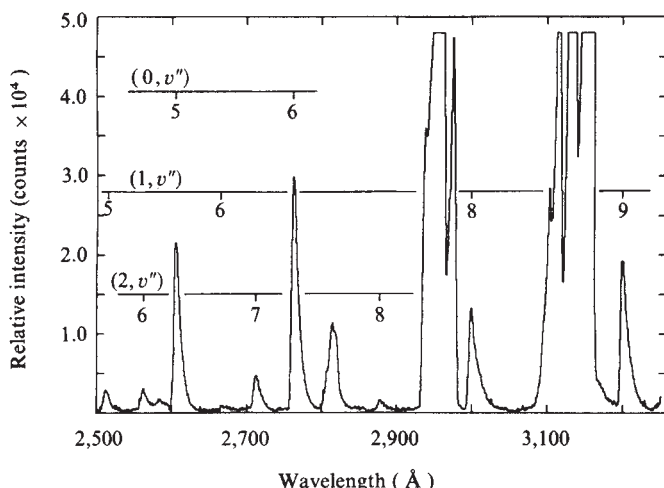


Fig. 1 Afterglow spectrum showing Vegard-Kaplan band [$\text{A}^3\Sigma_u^+ \rightarrow \text{X}^1\Sigma_u^+$] emission from the $v' = 0, 1$, and 2 vibrational levels.

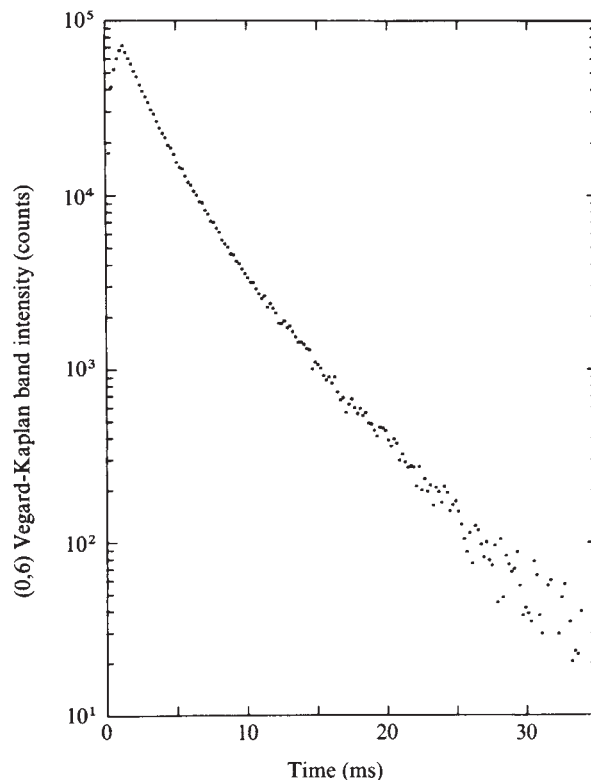


Fig. 2 The temporal behaviour of the (0, 6) Vegard-Kaplan band. The buildup of the $\text{N}_2(\text{A}^3\Sigma_u^+; v' = 0)$ concentration during the 1.0 ms microwave discharge can be seen readily. From the decay of the (0, 6) band intensity during the afterglow period, the total O_2 quenching coefficient for $v' = 0$ could be evaluated.

measuring the absolute intensity and the three-dimensional spatial distribution of the Vegard-Kaplan bands [$\text{A}^3\Sigma_u^+ \rightarrow \text{X}^1\Sigma_u^+$] (see Fig. 1). With a typical power pulse width of 200 μs and a repetition rate of 20 pulses per s, $\text{N}_2(\text{A}^3\Sigma_u^+)$ concentrations of $> 2 \times 10^{11} \text{ molecules cm}^{-3}$ were produced by the end of the discharge period in a mixture consisting of 6.0 torr argon, $120 \times 10^{-3} \text{ mm Hg}$ nitrogen, and $1.0 \times 10^{-3} \text{ mm Hg}$ oxygen. Pulse counting and coherent summing techniques were used to observe the temporal behaviour of the VK emission during the afterglow period. The experiment was generally allowed to accumulate photon counts until the decay of the VK bands originating from each of the $v' = 0, 1$, and 2 vibrational levels that were detectable could be followed over an intensity range of at least three orders of magnitude (Fig. 2). The absolute sensitivity of the entire optical system was determined using standard lamps (both tungsten ribbon and deuterium lamps) calibrated by the National Bureau of Standards and by the Optronics Laboratory. The absolute accuracy of the calibration of the optical system approaches that of the standard lamps themselves ($\sim 5\%$). From the time-dependent data, the quenching coefficients for the depopulation of $\text{N}_2(\text{A}^3\Sigma_u^+)$ in a specific vibrational level by O_2 , $k(v')$, could be evaluated. From these results [$k_0 = 1.9 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$, $k_1 = 4.0 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$] and from the absolute intensity and spatial distribution measurements, the total $\text{N}_2(\text{A}^3\Sigma_u^+)$ loss rate within the volume of the microwave cavity from reaction with and/or quenching by O_2

$$L(\text{A}^3\Sigma_u^+) = \frac{1}{\tau_0} \sum_{v'} k_{v'} n(\text{O}_2) \int_0^{\tau_0} \int_{\text{volume}} M_{v'}(\vec{r}, t) dt d\vec{r}^3 \quad (2)$$

was determined. In this expression, $M_{v'}(\vec{r}, t)$ is the local $\text{N}_2(\text{A}^3\Sigma_u^+; v')$ concentration at a specific time during the discharge or afterglow while $n(\text{O}_2)$ is the molecular oxygen density, and τ_0 is the power-pulse repetition period.

The buildup of N_2O as the result of reaction (1) was observed by a quadrupole mass spectrometer which sampled the gas

through a small aperture in the side of the cavity (Fig. 3). Typical N_2O steady-state concentrations of 2×10^{12} molecules cm^{-3} were observed. The limiting sensitivity of the mass spectrometer to N_2O in the conditions of the experiment was $\sim 10^9 \text{ cm}^{-3}$ so that accurate measurements of the N_2O concentration could readily be made. The N_2O signal was corrected for a small contribution ($<10\%$) due to carbon dioxide whose concentration could be determined accurately from the magnitude of the mass peak at 22 AMU. The latter is due almost exclusively to CO_2^+ ions and not N_2O^{2+} because the ratio of the effective ionization efficiency for these species in the conditions of the experiment was $\text{CO}_2^+/\text{CO}_2^+ = 1.29 \times 10^{-2}$ and $\text{N}_2\text{O}^{2+}/\text{N}_2\text{O}^+ < 5 \times 10^{-5}$, respectively.

In the present work, particular attention was paid to the potentially undesirable effects of impurities either in the gases used or as outgassing by-products from the vacuum system itself. To minimize these difficulties, the vacuum system was constructed entirely of stainless steel using metal gaskets where components were joined, and it was baked until the background pressure was 5×10^{-10} torr, and the virtual leak rate for the entire vacuum system was reduced to less than 10^{-10} torr s^{-1} . The neutral mass spectrometer was also used to determine the actual composition of the gas in the microwave cavity including other oxides of N_2 (NO and NO_2). These measurements showed quite clearly that the local $n(\text{O}_2)$ concentration in the microwave cavity was reduced significantly (15–20%) as the result of reaction (1), and it underlines the importance of measuring the absolute, *in situ* concentration of all reactants in a yield measurement.

The N_2O molecules formed by reaction (1) are removed from the microwave cavity chiefly by pump-out through the aperture servicing the mass spectrometer and, to a minor extent, by reactions in the discharge and afterglow periods. The total nitrous oxide loss rate, $(V/\tau_1)N$, when the discharge was on, was determined from the N_2O rise-time data (τ_1), the volume of the microwave cavity, V , and the N_2O concentration, N , measured by the mass spectrometer. The relationship between the steady-state N_2O and $\text{N}_2(\text{A}^3\Sigma_u^+)$ loss rates is

$$(V/\tau_1)N = \epsilon L(\text{A}^3\Sigma_u^+) \quad (3)$$

where ϵ is the efficiency of reaction (1). In conditions where τ_1 is kept constant, equation (3) predicts that the steady-state N_2O concentration will be directly proportional to the total number of $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules observed per cycle (equation (2)). This behaviour is shown quite clearly by the data of Fig. 4. By varying τ_0 , $n(\text{O}_2)$ and the microwave power-pulse width, the N_2O

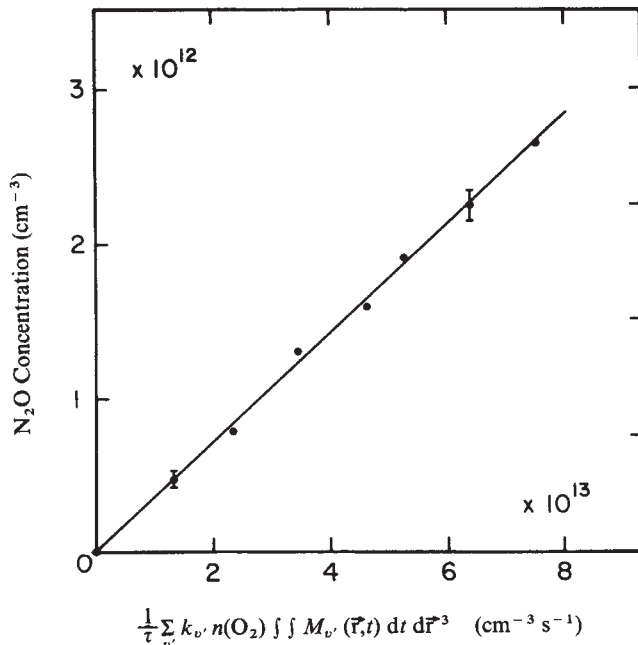


Fig. 4 A plot of the measured N_2O concentration versus the total $\text{N}_2(\text{A}^3\Sigma_u^+)$ loss rate.

concentrations predicted by equations (2) and (3) were further verified, and the efficiency, ϵ , was evaluated. The results of this study show that $\epsilon = 0.6$ with a probable absolute error of $\pm 30\%$.

The present study confirms the suggestion by Malcombe-Lawes³ that N_2O is formed efficiently when metastable $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules react with O_2 . Some nitric oxide was also observed in our experiment [$\text{NO} \sim 0.2 \text{ N}_2\text{O}$] possibly as the result of the reaction of N_2O with $\text{O}(\text{D})$ atoms formed in the microwave discharge. The observed NO_2 concentration was less than 10^{10} cm^{-3} even though NO_2^+ was a major afterglow ion. This suggests that the NO_2 buildup is rate-limited by ion-molecule reactions which rapidly convert NO_2 molecules to NO_2^+ ions that subsequently recombine dissociatively to form NO .

Recent rocket-borne studies of $\text{N}_2(\text{A}^3\Sigma_u^+)$ excitation in auroral substorms^{6–9} indicate that energetic electrons degrading in air at low pressure ($p < 10^{-3}$ torr) form $\sim 0.35 \text{ N}_2(\text{A})$ molecules per electron-ion pair. These results, when combined with the present study, indicate a minimum energy cost of $\sim 157 \text{ eV}$ per N_2O for $\text{A}^3\Sigma_u^+$ molecules in the $v'=0$ and 1 vibrational levels. The lower energy cost reported by Harteck and Dondes¹ (33 eV per N_2O) may be a consequence of the high pressures used in the neutron experiments. In the conditions of their experiment, many metastable and excited states which are formed copiously by particle bombardment and which relax radiatively in our afterglow experiment would be collisionally deactivated. This process might result in enhanced $\text{N}_2(\text{A})$ production (and thus N_2O) at atmospheric pressure or these species [for example, $\text{N}_2(\text{a}^1\pi_g)$] might react directly with O_2 to form nitrous oxide. Thus, when the A-state is formed by particle precipitation in auroral substorms, an energy cost of $\sim 157 \text{ eV}$ per N_2O is probably realistic. But, when the A-state is formed by X-ray and cosmic ray absorption in the stratosphere and below, a yield of one N_2O per electron-ion pair may be a better estimate.

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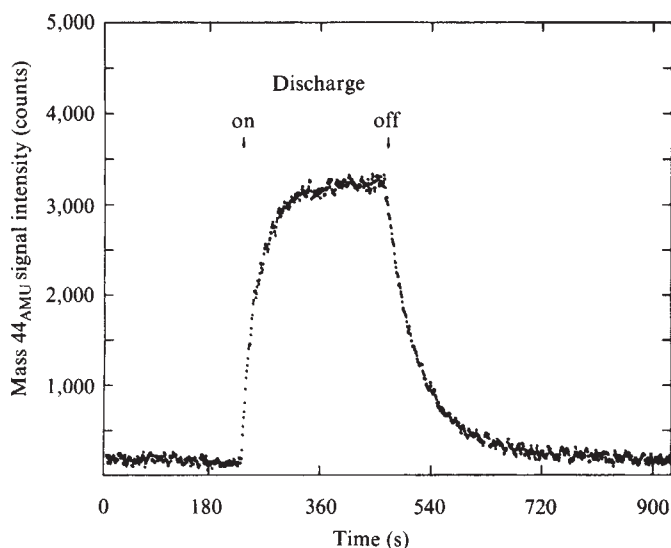


Fig. 3 Mass 44 AMU neutral mass spectrometer signal showing the buildup of nitrous oxide when the periodically pulsed microwave discharge is turned on and its subsequent pump-out when the discharge is turned off.

Production of nitrous oxide in the auroral D and E regions

E. C. Zipf

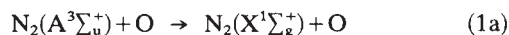
Department of Physics and Astronomy, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

S. S. Prasad

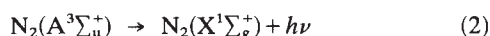
Jet Propulsion Laboratory, Pasadena, California 91103

Metastable $N_2(A^3\Sigma_u^+)$ molecules are formed efficiently in auroral substorms by electron-impact excitation of N_2 . In the auroral D and E regions, $N_2(A^3\Sigma_u^+)$ molecules react rapidly with O_2 to form N_2O at a globally averaged rate that could be as high as $2 \times 10^{27} s^{-1}$. We report here that in favourable substorm conditions, the N_2O density at 100 km could be as large as $10^9 cm^{-3}$.

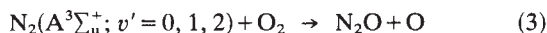
Metastable $N_2(A^3\Sigma_u^+)$ molecules are produced copiously in auroral substorms by the impact of low-energy electrons^{1,2}. Above 120 km, quenching by atomic oxygen



and radiation of the Vegard-Kaplan bands



are the dominant $N_2(A^3\Sigma_u^+)$ loss mechanisms, while below 100 km collisional deactivation by O_2 rapidly becomes the principal depopulation process^{3,4}. Because a recent laboratory study⁷ shows that the reaction



produces nitrous oxide with an efficiency of $0.6 \pm 30\%$, the auroral borealis will produce significant quantities of N_2O poleward of latitude 55° .

Detailed studies of the excitation of $N_2(A^3\Sigma_u^+)$ molecules in auroral substorms have provided some insight into the maximum N_2O production rate in an auroral arc¹⁻⁴. Although there is some disagreement concerning the absolute magnitude of the pertinent excitation cross-sections and the shape of the auroral secondary-electron flux, the recent work of Sharp *et al.*^{1,5,6} shows that the ratio of the $N_2(A^3\Sigma_u^+)$ production rate to the total ion-pair production rate, q , is ~ 0.35 . Thus, in view of the $N_2(A^3\Sigma_u^+)$ laboratory results, the N_2O production rate, $P(N_2O)$, could be as much as $0.21 q$. As the globally averaged energy deposition rate due to particle bombardment during periods of maximum solar activity⁸ is $\sim 6 \times 10^{29} eV s^{-1}$, the maximum N_2O production rate would be $\sim 4 \times 10^{27} molecules s^{-1}$ if reaction (3) was the only A-state loss mechanism.

Table 1 N_2O loss processes and chemical lifetimes at 100 km

	Reaction	Lifetime for IBC I aurora (s)
R(1)	$O(^1D) + N_2O \rightarrow 2NO$ or $N_2 + O_2$	1.1×10^7
R(2)	$N(^2D) + N_2O \rightarrow NO + N_2$	1.3×10^8
R(3)	$O^+ + N_2O \rightarrow O_2^+ + N_2$	1.0×10^9
R(4)	$N^+ + N_2O \rightarrow NO^+ + N_2$	3.6×10^7
R(5)	$N_2^+ + N_2O \rightarrow NO^+ + N_2 + N$	1.3×10^7
R(6)	$N_2^+ + N_2O \rightarrow N_2O^+ + N_2$	7.1×10^6
R(7)	$e + N_2O \rightarrow N_2O^+ + 2e$	1.2×10^8
R(8)	$e + N_2O \rightarrow$ dissociation products	1.2×10^8
R(9)	$N_2O^+ + O_2 \rightarrow O_2^+ + N_2O$	1.1×10^{-3}
R(10)	$N_2O^+ + e \rightarrow$ dissociation products	6.0×10^{-2}

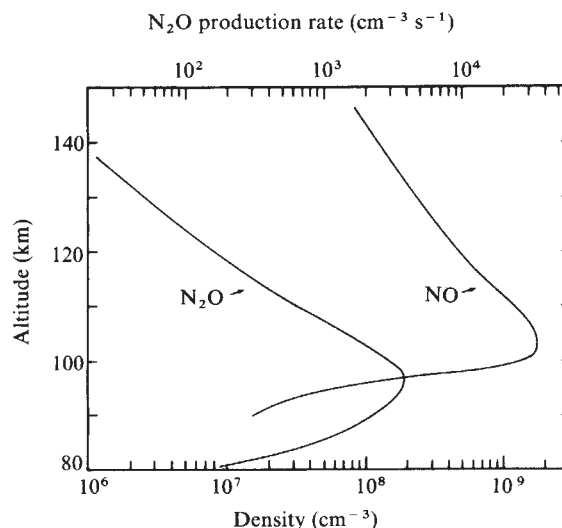


Fig. 1 The N_2O production rate in an IBC I aurora⁵ [$I(3,914 \text{ \AA}) = 4 \text{ kR}$]. The N_2O density assuming constant energy deposition for $5 \times 10^4 s$ is compared with the NO values calculated by Gerard and Rusch¹¹ for the same aurora and equal integration times.

Although reaction (3) does control the $N_2(A^3\Sigma_u^+)$ chemistry below 95 km, most of the energy deposited by precipitating auroral electrons is above 95 km where processes (1) and (2) are also quite important. Thus, the effective N_2O production rate will be somewhat less than the upper limit obtained above. A more realistic assessment can be made from *in situ* measurements of the volume emission rate of the (0, 0) first negative band of N_2^+ , $\eta(3,914 \text{ \AA})$, in typical IBC I and II⁺ aurora. This emission feature is frequently used to monitor the energy deposited in the auroral ionosphere by precipitating particles because its volume emission rate is directly proportional to the local ionization rate^{9,10},

$$\eta(3,914 \text{ \AA}) = \frac{q}{14.7} \frac{0.88[N_2]}{0.88[N_2] + 0.5[O] + [O_2]}$$

The local N_2O production rate is related to $\eta(3,914 \text{ \AA})$ by

$$P(N_2O) = 6.2 \eta(3,914 \text{ \AA}) \left\{ \frac{\epsilon k_3 [O_2]}{k_1 [O] + k_3 [O_2] + 0.52} \right\} \quad (4)$$

where $[O]$, $[O_2]$, and $[N_2]$ are the atomic oxygen, O_2 and N_2 concentrations, respectively, ϵ is the efficiency for producing N_2O by reaction (3), and k_1 and k_3 are the total reaction rate coefficients for $N_2(A^3\Sigma_u^+)$ deactivation by O and O_2 , respectively. Figure 1 shows the N_2O production rate calculated from published $\lambda 3,914 \text{ \AA}$ data for an IBC I aurora⁵ with k_1 , $k_3(v' = 0)$, and $k_3(v' = 1)$ equal to $1.2 \times 10^{-10} cm^3 s^{-1}$, $1.9 \times 10^{-12} cm^3 s^{-1}$, and $4.0 \times 10^{-12} cm^3 s^{-1}$, respectively^{5,7}. Table 1 lists the principal N_2O loss processes and the effective N_2O chemical lifetime at 100 km for each loss channel in the IBC I aurora studied by Sharp *et al.*⁵. None of these processes is an effective nitrous oxide sink on the time scale of a typical auroral substorm. The N_2O diffusion lifetime is also quite long (days). Thus, the local N_2O buildup in the auroral D and E regions is determined by source functions such as that shown in Fig. 1 integrated over the total amount of time that the auroral activity lasted (minutes to hours). As an example we have assumed in Fig. 1 that the energy deposition in the IBC I aurora remained constant for $5 \times 10^4 s$. The maximum predicted N_2O density, $2 \times 10^8 cm^{-3}$, is nearly an order of magnitude smaller than the NO concentration measured by Sharp¹² and estimated by Gerard and Rusch¹¹ assuming an equal integration period. With still longer integration times, the N_2O density would continue to increase relative to NO because the latter's growth is ultimately rate-limited by

the reaction $N + NO \rightarrow N_2 + O$. Thus, N_2O concentrations greater than 10^9 cm^{-3} could be produced in IBC III aurora or by lower-level activity lasting for many hours, and, in favourable conditions, the N_2O concentration could actually exceed the local nitric oxide density.

The total N_2O column production rate in Fig. 1 is $6.3 \times 10^9 \text{ cm}^{-2} \text{ s}^{-1}$ for an IBC I aurora in which the $\lambda 3,914 \text{ \AA}$ overhead intensity was 4.0 kR. These results imply an effective energy cost of $\sim 330 \text{ eV}$ per N_2O molecule in the aurora studied. Thus, a more realistic upper limit on the globally averaged N_2O production rate from auroral activity would be $\sim 2 \times 10^{27} \text{ s}^{-1}$. Table 1 implies that the chemical lifetime in the auroral *D* region is quite long. Thus, the buildup of N_2O in the polar region is probably rate-limited by transport processes. Although vertical diffusion will carry some of the nitrous oxide into the polar mesosphere, the horizontal winds which were invoked by Cravens *et al.*¹³ to explain the longitudinal distribution of NO in the *E* region, would also transport much of the N_2O equatorward where sunlight and reactions with $O(^1D)$ would slowly decompose it.

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Chemical evolution and ammonia in the early Earth's atmosphere

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A constraint on research into the evolution of the Earth's atmosphere may have been resolved by recent work on the photochemical reduction of atmospheric nitrogen by a naturally occurring form of TiO_2 (ref. 1). We show here that this source could have provided a small but vital biological supply of ammonia in the early atmosphere of the Earth. Locally the levels of NH_3 produced by this mechanism satisfy the requirements of origin-of-life biologists whilst global levels do not conflict with chemical and physical constraints on the surface/atmosphere system nor with geological evidence for early Precambrian conditions.

It has become increasingly difficult from the viewpoints of exobiology and astrogeology to harmonize theories on the evolution of the Earth's atmosphere. The problem centres around the existence and/or abundance of reduced constituents in the early atmosphere². Originally both astronomical and biochemical theories³ supported the hypothesis that the initial atmosphere of the Earth was composed primarily of chemically reduced compounds. However, Rubey's work⁴ and more recent geological (the oldest rocks yet discovered, from the Isua belt in West Greenland^{5,6}, are dated at (at least) 3,800 Myr and imply deposition conditions similar to the present-day) and

astronomical^{7,8} evidence argue strongly for a scenario in which degassing from a volatile-rich surface veneer leads to atmospheres composed mainly of carbon dioxide and water vapour (with smaller amounts of nitrogen and carbon monoxide) on all the terrestrial planets^{9–12}. The origin and subsequent evolution of life on Earth is easier to explain if a small mixing ratio of more reduced compounds such as methane and ammonia existed in the early Earth's atmosphere². Significant amounts of reduced gases could have been produced by the pyrolysis, during this early period of outgassing, of material similar to that still surviving in type I carbonaceous chondrites (survival of large quantities of intact organics during this process seems unlikely). However, a highly reducing atmosphere around the Earth, even if so produced, could not have persisted for any significant length of time¹³. The problem is particularly severe for ammonia. It has been argued that photodestruction of ammonia would have been substantially complete in $<1 \text{ Myr}$ (ref. 14). Kuhn and Atreya¹⁵ have concluded more recently that a mixing ratio $\leq 10^{-4}$ of ammonia would have been "irreversibly" converted to N_2 in $<40 \text{ yr}$. The photodestruction of NH_3 is an important problem as most models of chemical evolution require significant concentrations of NH_3 and NH_4^+ in solution for the synthesis of amino acids and other biochemicals. Bada and Miller¹⁶, for example, estimate that NH_4^+ concentrations must be $>10^{-3} \text{ M}$ to permit an equilibrium of equal concentrations of amino acids and the corresponding hydroxy acids at pH 8.

None of the studies of photolytic NH_3 destruction has considered the possible role of mechanisms for the resynthesis of NH_3 . The synthesis of HCN at high efficiency by means of spark discharges in mixtures of N_2 and CH_4 offers an attractive route to biologically important molecules^{17,18}. Aside from the question of the possible source and concentration of methane, clearly such syntheses may also involve the participation of NH_3 , although it is produced *in situ* through the decomposition of HCN in aqueous solution (always the major process at any reasonable concentration of HCN in water). Consequently, it is not clear whether NH_3 produced by this route alone in bodies of water on the Earth's surface would have escaped ultimate photolytic destruction, although reactions in liquid inclusions in frozen lakes could have been an exception¹⁹. However, this would not have been the only available pathway for the synthesis of NH_3 . Recent work on the direct photolytic reduction of N_2 on natural, semiconductor catalysts suggests a massive process which would have operated to increase steady state concentrations of NH_3 near ground level and, more importantly, in associated sources of ground water.

A systematic study of catalytic mechanisms of nitrogen fixation has established that titanium dioxide is an effective catalyst for the photochemical reduction^{20,21} of N_2 to NH_3 . A detailed investigation of naturally occurring areas rich in titanium revealed that certain deserts (such as the Imperial Sand Dunes, California) possess an extraordinary activity in the conversion¹ of atmospheric nitrogen to NH_3 . It is estimated that one acre ($\sim 4,047 \text{ m}^2$) of this type of desert sand generates between 1 and 10 kg of NH_3 in a year. Thus the current global production may be estimated at between 6.8×10^9 and $6.8 \times 10^{10} \text{ kg}$ per year. We suggest that this process would provide a significant abiotic source of ammonia on the primitive Earth. This source of NH_3 can only resolve the conflict between astronomers and biochemists if it supplies adequate amounts of this reduced gas to localized areas whilst not perturbing the surface/atmosphere system (especially the surface temperature and chemical nature of the troposphere) beyond the fairly close constraints imposed by geological data. Our results suggest that both these sets of constraints are satisfied by this source of ammonia. The rate of nitrogen reduction is obviously a function of the total area of a suitable catalyst and the type of irradiation that it suffers. It seems reasonable to assume that early in the history of the Earth the level of surface irradiation by UV light was considerably increased. Even if, as has been suggested²², the oxygen content of the atmosphere was within a factor of 10 of its present level 2,000 Myr ago, the earliest stages of surface/atmospheric

Table 1 The greenhouse effect caused by ammonia in the early Earth's atmosphere for the addition of 10^{13} kg NH_3

Time (Myr BP)	$p\text{CO}_2$ (mbar)	10^{13} kg NH_3 ΔT (K)
4,250	310	17.2 (327.2)
3,500	70	15.1 (311.1)
2,500	18	13.7 (305.7)

Temperatures (in parentheses) are values from ref. 11 with the NH_3 increment added.

interaction probably included a significant effect of NH_3 fixation by desert sands, particularly as near-UV light is also effective²⁰. Note that the measured production rates in these experiments indicate a substantially greater rate for the surface-catalysed production process than for the noncatalytic reverse oxidation. Thus a net accumulation of NH_3 in the vicinity of the catalyst would have been inevitable. We calculate, on the basis of an experiment described by Schrauzer¹, that the ammonia produced would have corresponded to a solution 0.01 M in the absorbed water of the desert sand tested. Thus the minimum requirements for the origin and subsequent evolution of life can be achieved, at least, locally. (The NH_3 mixing ratio in equilibrium with this concentration is pH dependent, but could not exceed 10^{-4} .)

However, are likely global levels of NH_3 consistent with the recognized climatological and geological data? We suggest that an upper limit for the global level of NH_3 , assuming a production rate enhanced by two orders of magnitude together with a residence time against chemical destruction in the atmosphere of between 10 and 100 yr, is 10^{13} kg in the early atmosphere of the Earth. Higher levels than this seem unlikely for several reasons: (1) ammonia dissolves readily in water and thus a large mixing ratio of NH_3 in an atmosphere known to be in contact with considerable areas of surface liquid water throughout the Precambrian²³ is difficult to sustain; (2) photochemical oxidation of NH_3 rapidly removes it from the bulk atmosphere; (3) surface temperatures are a direct function of the abundance in the atmosphere of any gas which possesses IR absorption bands. Ammonia's strong absorption in the IR, caused earlier speculations concerning the atmosphere of the early Earth to include relatively large mixing ratios of ammonia²⁴. There is clearly an important relationship between the likely production rates, the removal by photolysis and global temperatures.

The addition of even small amounts of a gas which possesses strong features in the IR part of the spectrum to atmospheres which are already in equilibrium with a surface at a temperature close to the present-day value (between 285 and 290 K) may pose serious problems to any atmosphere/surface evolution model. This is why we have computed the extra 'greenhouse effect' due to addition of 10^{13} kg of ammonia to the atmosphere. The precise global level of ammonia is extremely difficult to estimate as the destruction rate depends on the ground level mixing ratio, the eddy diffusion coefficient and the opacity of the atmosphere at the appropriate wavelengths^{14,15}. However, this new source seems to be of the same order of magnitude as previously computed destruction rates¹⁵ and thus the global budget of NH_3 could be enhanced. Geological evidence pertaining to the ambient surface temperatures^{5,23} is now an important source of information. The extra temperature increment has been calculated using computer programmes described in detail elsewhere²⁵. The numerical model includes the major regions of NH_3 band absorption (that is, 2.8–3.2; 5.5–7.2; 8.0–15.0 and longwave of 35.0 μm) plus the effects of broadening both by neutral gases and other absorbers. The solution of the radiative transfer equation is approximate but the accuracy and usefulness of the model over evolutionary time scales has been demonstrated^{10,26}. Particular attention has to be paid to the regions of overlap between absorption bands (in this case the bands of water vapour and ammonia). We have followed Goody²⁷ in taking the transmission in regions of over-

lap as the product of the fractional transmissions of the individual bands. This is more appropriate than the use of an empirical relationship for application to atmospheric evolution over geological time periods. The variation in the solar luminosity, planetary flux factor, albedo and partial pressure of all atmospheric constituents is included. Table 1 lists the 'greenhouse increments' for the addition of 10^{13} kg of ammonia to an evolving atmosphere of CO_2 with the present-day level of water vapour. (The effect of adding this amount of NH_3 to atmospheres more recently than 2,500 Myr ago has not been calculated as there are conclusive geological data against such high levels²⁸ and the irradiation of the surface by UV light would by this time have decreased considerably, thus reducing the fixation rate.) This carbon dioxide atmosphere is described in detail elsewhere¹¹. The resulting surface temperatures (that is, the temperatures of their model plus our calculated increment) are shown in parentheses in Table 1. (There is an interesting feedback effect associated with this mechanism for abiological nitrogen fixation—if surface temperatures rise in response to the addition of NH_3 to the early atmosphere then it is possible that larger areas of desert sands suitable for the catalytic reaction described by Schrauzer¹ may occur, thus producing a positive feedback cycle.) There are, of course, other climatological parameters which could be perturbed by such changes. The most difficult of these to consider is the possible perturbation in cloud cover. It has been suggested²⁹ that over geological time periods the percentage cloud cover may have remained approximately constant.

The computed average global surface temperatures in Table 1 seem to be rather high. Compare the value at 4,250 Myr ago with the early temperature of Venus calculated from the simple radiation balance equation

$$\frac{S}{f}(1-A) = \sigma T_e^4 \quad (1)$$

where S is the solar flux (Wm^{-2}), f the planetary flux factor²⁶, A the albedo, and σ the Stefan-Boltzman's constant.

At the time of planetary formation and assuming an increase in solar luminosity of between 40 and 45%, the effective temperature of Venus from equation (1) is between 295 K (for $f=4$) and 350 K (for $f=2$) (ref. 10). It is generally believed that the high early surface temperatures on Venus triggered the runaway greenhouse effect which finally resulted in the present-day massive atmosphere and very high surface temperatures (~ 750 K). Our results suggest, therefore, that 10^{13} kg of NH_3 in the evolving atmosphere of the Earth could produce surface temperatures too high for the subsequent evolution that is known to have occurred. The question of the possibility of a climate catastrophe has been discussed recently^{28,30}. We suggest that the geological data^{23,31} do not support any scenario which has either very high early surface temperatures or very large mixing ratios of ammonia. Note, however, that work on oxygen isotope levels in certain cherts suggests higher surface temperatures in the Precambrian³². We consider that surface temperature data are a valuable additional source of information. If the primitive atmosphere²⁸ contained even larger amounts of NH_3 (say through a combination of catalytic fixation and impact degassing of CI type material), the known surface temperature evolution would seem to be impossible. For instance, we have computed the additional greenhouse increment for 10^{15} kg NH_3 in the 4,250 Myr BP atmosphere in Table 1. In this case the value of $\Delta T = 108.3$ K gives a global average temperature of 418.3 K.

The present results suggest that a source of abiological ammonia by fixation of atmospheric N_2 in the presence of TiO_2 may provide enough reduced gas for local conditions (as dissolved NH_3) to be hospitable for the origin and subsequent evolution of life. However, the strong dependence of global surface temperatures on the partial pressure of ammonia indicates that the global mixing ratio is unlikely ever to have been above 1.5×10^{-5} by volume. This mechanism of NH_3 production may have constituted the major source of NH_3 in the prebiotic world, although photochemical destruction clearly

restrained the build-up of ammonia levels in the primitive atmosphere.

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Does the whole of the Earth's core convect?

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Higgins and Kennedy¹ used data on the behaviour of iron at high temperatures and pressures to infer that the top of the Earth's liquid core is stably stratified. Attempts to confirm this result both thermodynamically² and using seismological data³ have been inconclusive. I present here geomagnetic results which may resolve the controversy. If a stratified region exists there will be no upwelling or downwelling of core fluid at the core-mantle boundary (CMB), so there will be no horizontal divergence of velocity $\mathbf{v}$, that is $\nabla_{\mathbf{H}} \cdot \mathbf{v} = 0$, where

$$\nabla_{\mathbf{H}} = \nabla - \hat{\mathbf{r}}(\hat{\mathbf{r}} \cdot \nabla)$$

$\hat{\mathbf{r}}$ denotes the unit radial vector. This hypothesis can be tested directly, using geomagnetic data, at a few isolated points on the CMB, and local averages of $\nabla_{\mathbf{H}} \cdot \mathbf{v}$ can be examined over the rest of the surface. A statistical treatment of the results strongly suggests that $\nabla_{\mathbf{H}} \cdot \mathbf{v} = 0$, which is a consequence of a stably stratified layer.

The radial induction equation at the CMB, where $\mathbf{v} \cdot \hat{\mathbf{r}} = 0$, is, on the decade time scale^{4,5},

$$\dot{B}_r + \mathbf{v} \cdot \nabla_{\mathbf{H}} B_r + B_r \nabla_{\mathbf{H}} \cdot \mathbf{v} = 0 \quad (1)$$

where a dot denotes time differentiation and the subscript r the radial component. This equation cannot be used to calculate the horizontal divergence of velocity directly because of a fundamental ambiguity^{4,5}. However, at local extrema of B_r (points at which $\nabla_{\mathbf{H}} B_r = 0$), the second term of equation (1) vanishes, eliminating the unknown $\mathbf{v}$. If the horizontal velocity divergence is zero, these extrema must be points at which $\dot{B}_r$ is

Table 1 Secular variation and horizontal divergence at extrema

Position (θ° , ϕ°)	$\dot{B}_r (\times 10^{-3}) \gamma \text{ yr}^{-1}$	$\nabla_{\mathbf{H}} \cdot \mathbf{v} (\times 10^{-3}) \text{ yr}^{-1}$
(9, 329)	-0.78	-17.46
(34, 110)	-0.04	-0.06
(37, 45)	-0.71	5.73
(39, 268)	-0.45	-0.69
(48, 357)	-0.32	-0.99
(52, 164)	0.35	7.63
(65, 59)	-2.10	-4.13
(81, 190)	0.75	2.25
(94, 350)	-0.68	-1.66
(98, 33)	-2.34	-3.87
(103, 84)	2.85	5.53
(110, 267)	1.18	5.04
(118, 176)	0.43	-1.13
(123, 351)	0.09	-15.01
(128, 48)	-0.16	-0.60
(138, 295)	1.21	4.42
(145, 348)	0.95	5.76
(148, 113)	1.00	1.70
(163, 225)	1.76	2.57

zero. Roberts and Scott⁴ have observed that this condition would hold if the velocity were purely toroidal.

The analysis was carried out for the International Geomagnetic Reference Field (IGRF) model⁶ of 1965.0, a set of surface spherical harmonic coefficients up to eighth degree and order. The radial component of the field at the CMB ($r = 3,485 \text{ km}$) is shown in Fig. 1. Solid contours indicate positive and zero values and dotted contours are negative, the contour interval is 1 G. The dashed lines are contours of zero $\dot{B}_r$, and in general they pass remarkably close to the extrema of B_r . I have found 19 extrema, marked by crosses in Fig. 1, whose secular variation and horizontal velocity divergence are given in Table 1. It is impossible to construct satisfactory error bounds either for the positions of the extrema or the zero $\dot{B}_r$ contours, so, to check that these numbers are significant, I have compared the mean and standard deviation of these $\dot{B}_r$ values with those from sets of 19 geographically randomly distributed points (see Table 2). None of these sets contained points with an angular separation of less than 15° . Clearly, the random points are more variable: none of the 24 sets had a smaller standard deviation than that of the extremal points. Taking all the random points together (mean 0.26, standard deviation 2.05), I used a one-tailed F -test on the ratio of the variances of the random and extremal points. The null hypothesis of the variances being equal can be rejected

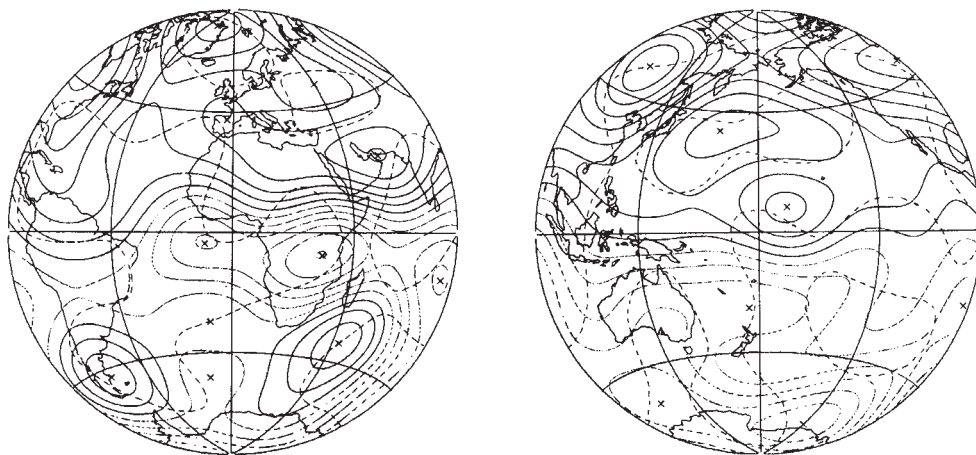
Table 2 Means and standard deviations of $\dot{B}_r$ for extremal and random sets of points

Mean ($\times 10^3$)	Standard deviation ($\times 10^3$)
0.157	1.251†
0.442	1.520
0.493	1.522
0.932*	1.527
0.096	1.530
0.053	1.563
1.003*	1.578
0.005	1.582
0.577	1.589
-0.142	1.589
0.467	1.655
0.128	1.667
-0.281	1.815
-0.187	1.820
0.642	1.913
0.414	1.935
0.267	1.992
-0.308	1.997
0.144	1.998
0.468	2.016
0.392	2.020
0.357	2.051
-0.227	2.087
0.235	2.288
0.246	2.312

* The mean is inconsistent with zero at 95% level (using a 2-tailed Student's t -test).

† Extremal set; the rest are randomly generated.

Fig. 1 Radial component of main field for 1965.0 at CMB on a Lambert equal area projection are positive and zero values, dotted contours negative. Dashed lines are contours of zero radial secular variation. Local extrema of the radial main field component are marked by crosses.



at the 99% level. Looking at the means of the individual sets, five have a smaller mean than that of the extremal points, but two others (marked with an asterisk in Table 2) are inconsistent with zero at the 95% level (using a two-tailed Student's *t*-test). Thus the values of $\dot{B}_r$ (and hence $\nabla_H \cdot \mathbf{v}$) at the extrema are significant—they are consistent with zero and less variable than would be expected from purely random sampling.

Backus (personal communication) suggested that $\nabla_H \cdot \mathbf{v}$ over the rest of the core could be estimated by integrating equation (1). The most useful form is shown to be, after some manipulation,

$$\iint_{S_0} \dot{B}_r dS + B_r \iint_{S_0} \nabla_H \cdot \mathbf{v} dS = 0 \quad (2)$$

where the surface S_0 is bounded by a contour of B_r . Thus an additional necessary (but not sufficient) condition is that integrals of $\dot{B}_r$ over any surface bounded by a contour of B_r must vanish if $\nabla_H \cdot \mathbf{v} = 0$. I have computed surface integrals for the 1965.0 IGRF with 10% accuracy; the corresponding values of $|\iint \nabla_H \cdot \mathbf{v} dS| / \iint dS$ are presented in Table 3. Each surface of integration encompasses 1 G variations in the main field (2 G between +1 and -1 G), so the surfaces of integration are independent and together cover the whole core. Again the effect of errors in the spherical harmonic coefficients cannot easily be assessed, so the results were compared with sets of random integrals of $\nabla_H \cdot \mathbf{v}$. The random integrals were generated by rotating the secular variation field, keeping the main field fixed, and then integrating $\dot{B}_r$ over the same surfaces. This preserved the frequency content of the secular variation field.

The absolute values of the integrals of $\nabla_H \cdot \mathbf{v}$ per unit surface area for the rotated secular variation fields are also given in Table 3. The two rotations were through 180° in longitude (the transformation $(\theta, \phi) \rightarrow (\theta, 180^\circ + \phi)$) and North to South Pole inversion $((\theta, \phi) \rightarrow (180^\circ - \theta, 180^\circ - \phi))$. Of both sets of random integrals 60% are larger than for the unrotated $\dot{B}_r$ field, and the mean integral of $\nabla_H \cdot \mathbf{v}$ per unit area, regardless of sign, is 62.3 and 38.8 after rotation, but 30.4 for the original integrals. These results suggest that the average horizontal divergence is less than for a random secular variation field.

The results presented here should be relatively insensitive to errors in the IGRF because a statistical treatment has been adopted, but there are problems associated with the downward continuation of the surface fields. The short-wavelength crustal component of the main field and errors in the secular variation are amplified by extrapolation to radii below the surface. Most secular variation models are dominated by small-scale instabilities at the CMB. A fuller report incorporating techniques for reducing these problems will be given elsewhere⁷.

Assuming $\nabla_H \cdot \mathbf{v} = 0$ everywhere, the velocity component perpendicular to contours of B_r can be found everywhere on the CMB. An upper limit for the vertical growth rate of velocity, $\partial \mathbf{v} / \partial r$, at the CMB is $1.44 \times 10^{-10} \text{ s}^{-1}$ (averaging $|\nabla_H \cdot \mathbf{v}|$ from Table 1). Benton and Muth⁸ recently obtained $1.23 \times 10^{-10} \text{ s}^{-1}$

and $0.95 \times 10^{-10} \text{ s}^{-1}$ using independent methods. Benton⁹ has developed a method similar to part of that described here. The data say nothing about the region below the CMB and convection may still occur deeper down. An alternative explanation of this result is that the core fluid flow is dominated by rotation (geostrophic) and is uninfluenced by strong magnetic fields.

Table 3 Integrals of $\nabla_H \cdot \mathbf{v}$ over surfaces bounded by contours of B_r for the original and rotated B_r field

		$ \iint \nabla_H \cdot \mathbf{v} dS / \iint dS (\times 10^{+4})$		
B_r (G)	Location	Original field	After 180° rotation	North to South Pole inversion
>7	Russia	1.54	36.28	42.53
6-7	Russia	3.79	35.78	43.08
>6	North America	5.16	6.13	39.29
5-6	Russia	7.19	35.52	47.48
5-6	North America	2.66	7.48	25.32
>5	Persian Gulf	40.79	523.03	16.20
4-5	Russia	11.72	31.70	44.85
4-5	North America	2.01	11.59	12.33
4-5	Persia	36.10	14.00	1.10
3-4	Asia	23.80	0.32	10.03
3-4	North America	24.06	19.81	9.39
>3	Mid-Pacific	24.84	61.64	6.95
2-3	Northern Hemisphere land masses	6.10	2.69	10.00
2-3	Mid-Pacific	38.00	62.62	110.42
>2	Off South Africa	13.04	217.71	26.79
>2	South America	54.47	33.15	64.76
1-2	Equatorial	74.14	28.50	4.45
1-2	North Pole	8.33	11.37	52.68
1-2	Off South Africa	69.11	292.33	71.80
1-2	South America	105.61	70.22	133.24
<1	North Pole	36.47	62.84	7.65
<1	North Pacific	60.72	53.09	205.58
-1+1	Equatorial	59.85	81.49	107.66
-1+1	South Atlantic	76.70	112.54	49.31
-2-1		13.83	94.55	25.77
-3-2		14.97	57.89	13.16
-4-3		1.70	7.65	26.79
<-4	West African Coast	17.21	6.24	9.35
-5-4	Central Africa	46.50	47.47	18.14
-5-4	Indian and South Pacific Oceans	3.53	18.18	5.65
<-5	Indian Ocean	55.70	7.46	9.77
-6-5	Central Africa	40.31	30.55	14.37
-6-5	Antarctic	4.85	18.08	9.90
<-6	Central Africa	40.54	25.35	12.23
<-6	Antarctic	37.02	38.11	8.88
Mean		30.37	62.29	38.44

The values of B_r indicate the contours that bound the surfaces of integration.

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Accumulation of calcic plagioclase in ocean-ridge tholeiite: an indication of spreading rate?

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Basaltic magma generated at the Mid-Atlantic Ridge (MAR) appears on eruption to be characterized by the widespread accumulation of calcic plagioclase (An_{70-90}) independently from olivine and clinopyroxene^{1–5}. Plagioclase accumulation is curiously absent from basalt formed at faster-spreading ridge axes such as the East Pacific Rise (EPR)^{6–11}, and it is interesting to speculate about the possible relationship of this phenomenon to spreading rate. Recent experiments¹² and studies of thermal regime at ocean-ridge spreading axes as a function of spreading rate^{13,14} suggest an attractively simple solution to the problem, and point the way to a useful genetic spreading rate distinction for ophiolite rocks of unknown provenance.

The major-element chemical variation of basalts sampled from the Atlantic and Pacific Oceans is depicted schematically in Fig. 1 showing principal mechanisms of post-genetic fractionation identified from least-squares analysis of rock and glass data (1–4). Trends 1–4 are all encountered in Atlantic basalts, but trend 3 (representing cotectic fractionation of plagioclase + clinopyroxene ± olivine) is the prevalent, if not exclusive, trend in Pacific basalts^{7–10}, with only one recently reported example of trend 1 (ref. 11). Trends 1 and 2 are rarely encountered in Atlantic basalts, but trend 4 is common. However, it is noticeably absent from the Pacific. This trend reflects accumulation of calcic plagioclase in both primitive (MgO-rich) and evolved (MgO-poor) liquids, and is interesting in view of experimental indications^{15–18} that plagioclase is at no pressure the sole anhydrous liquidus phase for tholeiitic basalt. Appeal to hydrous melting and high oxygen fugacity as a means to enrich primary magma in normative plagioclase^{2–4,15} is probably not justified in view of the low volatile contents measured in ocean-ridge basalts^{19,20} and ubiquitous FeO-enrichment trends in MORB glass²¹, and we are led to interpret it as an effect of differential phenocryst distribution under anhydrous conditions.

Density relations at one atmosphere are quite complex. Evolved, FeO-rich, basaltic liquid is more dense than MgO-rich primitive liquid²² while CaO-rich plagioclase, typically crystallizing from primitive magma, is denser than NaO-rich plagioclase characteristic of evolved magma¹². In general, however, most plagioclase varieties will be denser than primitive or moderately primitive magmas at pressures less than about 1 kbar (ref. 12). Slow cooling and crystallization in shallow reservoirs would allow plagioclase to accumulate along with denser mafic phases by gravitative separation from the liquid phase. Fujii and Kushiro¹² demonstrate that with increasing pressure

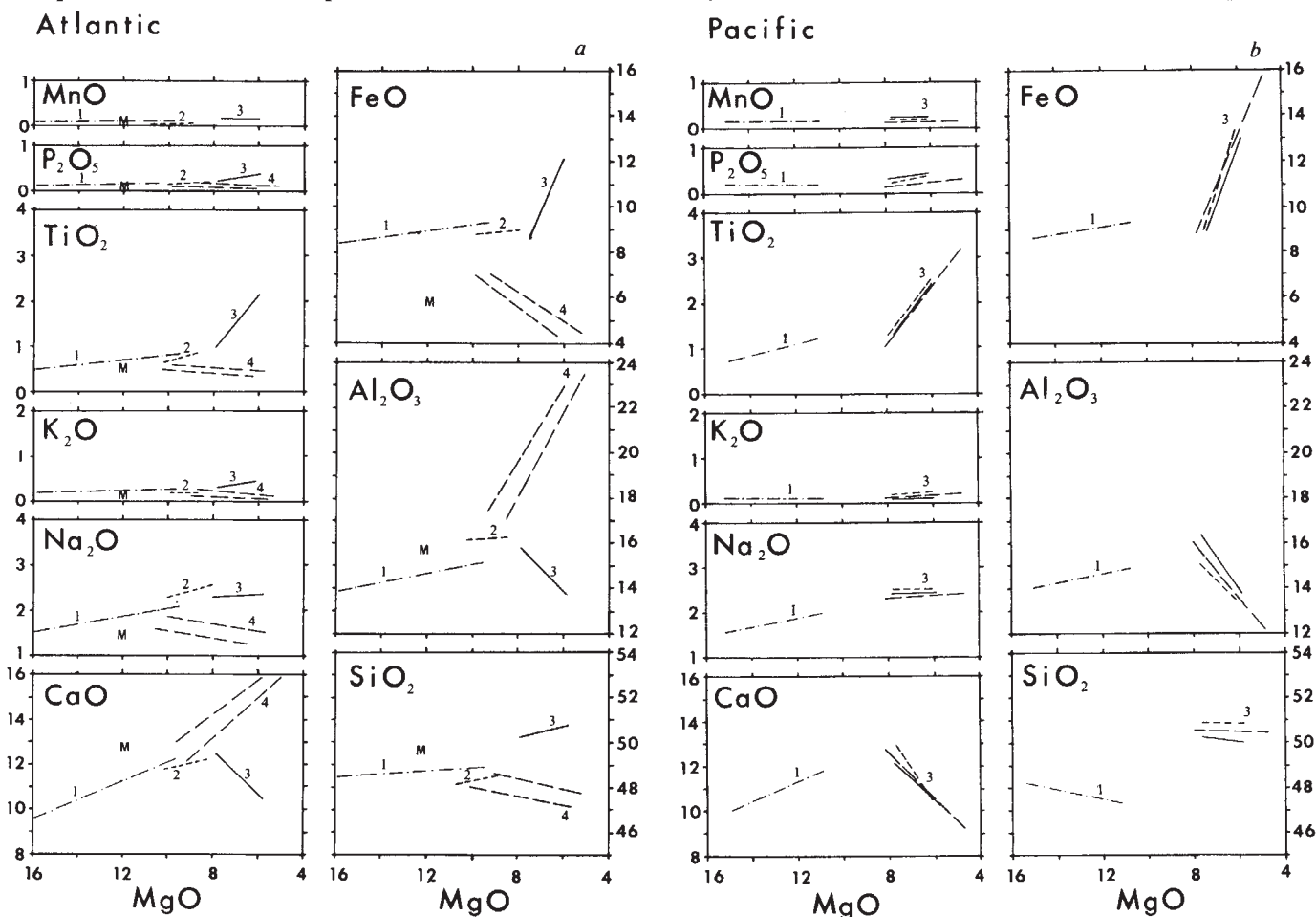


Fig. 1 MgO variation diagrams to illustrate ocean-ridge basalt fractionation, from data cited in *DSDP Initial Report* Vols 34, 37, 45, 46, 51–53, 64 and 65, and in refs 1–11, 21, 30. Trend lines as follows: (1) olivine control by fractionation or accumulation; (2) olivine + plagioclase fractionation; (3) plagioclase + clinopyroxene ± olivine fractionation; (4) accumulation of plagioclase; (M) accumulation of plagioclase + clinopyroxene + olivine. All types appear in Atlantic basalts; type 4 is rare or non-existent in Pacific basalts.

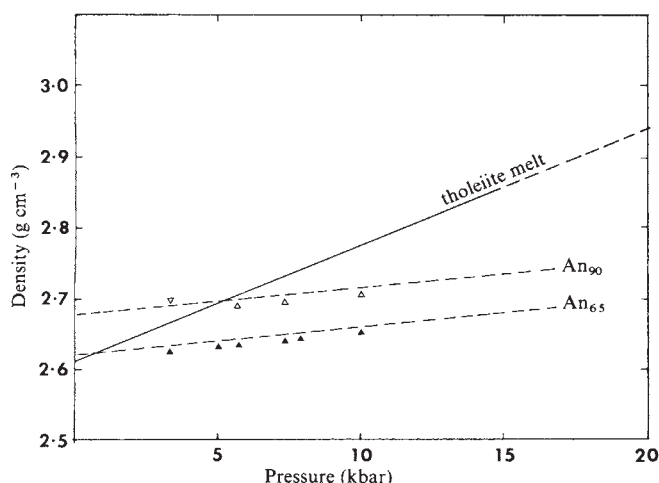


Fig. 2 Density versus pressure relations for Kilauea 1921 olivine tholeiite melt and plagioclase feldspar (after Fujii and Kushiro¹²). Solid lines indicate melt density and broken lines plagioclase density at near-liquidus temperatures. Triangle apices show direction of plagioclase movement.

there is a tendency for plagioclase to float in MgO-rich basaltic liquid, the extent of this tendency depending on plagioclase anorthite content and a significant increase in melt density with pressure. Fujii and Kushiro show that the melt density increase transcends that of plagioclase (An₉₀) at about 6 kbar (Fig. 2), indicating that pressure is the main requirement for plagioclase flotation. These relations may be confirmed by molar volume calculations²³ and directly through experiment¹².

By implication, therefore, the demonstration of plagioclase flotation independently from mafic phases in erupted basalt may reflect pressure-characteristic fractionation in ocean-ridge magma supply systems. The type and extent of fractionation resulting from the cooling and crystallization of mantle-released magma is determined by the interrelation in *P-T* space of basalt liquids, ocean-ridge geotherm and magmatic ascent path(s). Experimental evidence¹⁶ and the discovery of both spinel- and plagioclase-lherzolite 'fossil' melt residues^{24,25} suggests primary ocean-ridge tholeiite is generated by fairly advanced (15–25%) partial melting, at consistently low water and oxygen fugacities, near the solidus intersection of the plagioclase/spinel-lherzolite transition, that is, at about 1,300 °C and 8–10 kbar pressure. These conditions imply primary liquid compositions with 10–12 weight % MgO, consistent with requirements (for example, olivine compositions of ~Fo₉₀) for equilibration with common residue lherzolite compositions²⁶. More MgO-rich primary compositions²⁷ would show multiple saturation at about 15 kbar, so that a 'pivotal' region of 10–15 kbar (30–45 km depth) may be defined as a constraint to average ridge axis geotherm configurations (see ref. 28).

Sleep¹⁴ computed isotherm distributions for the upper 10 km of ridge axes in relation to spreading rates and was able to define the relative stability of magma reservoirs. According to Sleep's model, basalt supraliquidus temperatures become increasingly localized along axial geotherms as spreading rate declines. At very slow rates (<1 cm per yr), high-level magma storage appears unlikely and diapiric solid intrusion to the sea floor may occur. These considerations are combined schematically in Fig. 3, and together allow us to postulate:

- (1) Two or more stages of crystallization (for example, at 15–25 km and <5 km depth, but depending on model parameters) for magma ascending at slow-spreading (<about 5 cm per yr) axes; and
- (2) Exclusively low pressure (for example, < about 3 km depth) crystallization at fast-spreading (> about 5 cm per yr) axes, with direct ascent of superheated primitive magma into

shallow axial chambers; as general conditions for the steady-state geotherms depicted.

Although melting and magma uprise are probably episodic (constraints and limitations of this model are discussed in ref. 29), an important qualitative deduction can be made from Fig. 3: that polybaric fractionation, a regime promoting the accumulation of plagioclase in magma, is far more likely at slow-spreading than fast-spreading ridge axes. Further support for the influence of spreading rate on fractionation is provided by the widespread development of phenocryst reaction morphologies in Atlantic, but not in Pacific, basalts^{6–10,30–33}. Resorption and oscillatory zoning of plagioclase and clinopyroxene phenocrysts are often attributed to minor variations in P_{H_2O} and/or magma mixing³⁴, but are equally explicable as due to partial polybaric re-equilibration of higher-pressure liquidus assemblages in cogenetic liquid, during its storage at low pressure.

'Fast-spreading' basalts are invariably aphyric or only sparsely phyrical^{7–10}. There is no reported occurrence of phenocryst reaction of the type observed in Atlantic basalts, or of plagioclase accumulation. Where present, phenocrysts appear to represent low pressure liquidus crystallization products^{7–10}. Interestingly, basaltic crust generated at 'intermediate' spreading rates (for example, at the Gorda Rise, Costa Rica Rift and Gulf of California) shows incipient development of the 'slow-spreading' petrographic tendencies⁶. Finally, intra-flow variation due to plagioclase accumulation during post-eruptive phenocryst movement (flow differentiation) is not significant in comparison to that associated with pre-eruptive fractional crystallization³⁵, and in any case could not be spreading rate-dependent.

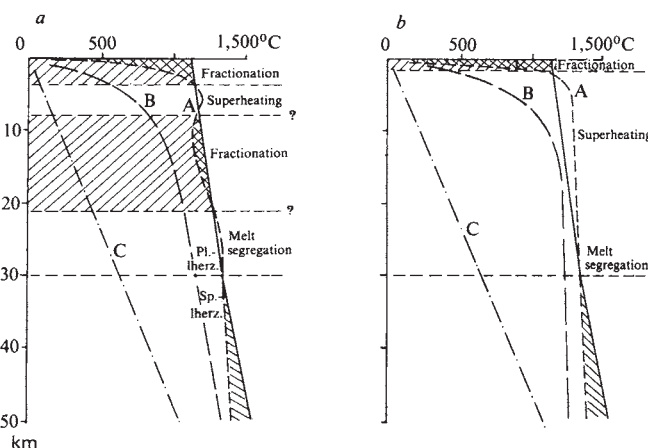


Fig. 3 Thermal gradients interpolated from Sleep's¹⁴ computed isotherm distribution for *a*, a slow-spreading (1 cm per yr) ocean-ridge and *b*, a fast-spreading (5 cm per yr) ridge, and from experimental data for high pressure 4-phase saturation in primitive tholeiite (ref. 16); *a*, at spreading axis, *b*, at 30 km from axis, and *c*, 'average' ocean crust. Schematic depth intervals for fractionation, superheating and melt segregation reflect idealized steady-state spreading. Pl. lherz., plagioclase-lherzolite; Sp. lherz., spinel-lherzolite.

To summarize, I interpret the accumulation of plagioclase in ocean-ridge tholeiite as a comparatively high pressure effect. It is characteristic of slow-spreading axes such as the MAR for which thermal models^{13,14} and seismic data^{36,37} imply transient, polybaric, fractionation systems, while it is incipient only, or absent, at faster-spreading axes. The oft-cited general enrichment of FeO*, TiO₂ and lithophile elements at the latter (for example ref. 38), attributed to iterative fractionation processes under low f_{O_2} conditions in steady-state magma chambers^{39,40}, may be in part illusory, and result simply from relative dilution of these components by the accumulated plagioclase in Atlantic

basalt. As further confirmation, no systematic chemical distinction of basaltic glass (that is liquid fraction) can be made between fast and slow-spreading axes²¹.

Rare earth element and Sr data should be sensitive to plagioclase accumulation. However their interpretation has to consider the combined potential effects of plagioclase and apatite (?) buffering in the melt residue, and removal of plagioclase during fractional crystallization prior to accumulation. It should also be noted that Eu anomalies are comparatively slight for CaO-rich plagioclase in relation to basalt liquid at low f_{O_2} (refs 41, 42). DSDP/IPOD basalts⁴³⁻⁵¹ show correspondence between coarsely plagioclase-phyric lithologies and small (but significant) positive Eu anomalies, and decreased Rb/Sr, Zr/Sr, Ti/Sr (etc.) ratios as compared to glass or aphyric types.

Evidence for phenocryst-liquid reaction and plagioclase accumulation may be a sensitive indicator of thermal and kinematic conditions at dilating plate margins, and provide important information on the nature of magma supply at ocean ridges. Such petrochemical spreading rate discriminants may be applicable to obducted ocean crust whose conditions of formation at extinct accretionary plate margins are not easily inferred from other criteria.

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Behaviour of phosphate in estuarine water

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The phosphate load in many rivers has been increased through soil disturbance and discharge of sewage and other wastes. Understanding how much phosphate may be removed from the waters of rivers and estuaries to the sediment would aid waste disposal planning. Phosphate carried through estuaries to coastal waters can increase growth of phytoplankton and macroalgae, with effects on fisheries and fouling of structures and beaches. Removal of dissolved phosphate to the sediment in estuaries has been predicted from indirect evidence¹⁻³, but measurements of dissolved phosphate in estuaries have shown various patterns of behaviour⁴⁻⁷. A constant level of dissolved phosphate over a wide salinity range, observed in some estuaries^{5,7}, has been attributed to buffering of the phosphate by particulate matter. This implies the release of phosphate from particulate matter in some conditions. Seawater held in contact with large concentrations of sediment in laboratory conditions tends to a steady-state level of dissolved phosphate in the range 3-28 $\mu\text{g P l}^{-1}$ (refs 8-11). Our laboratory studies, using a simple estuarine mixing model system, show that inorganic removal of phosphate is likely to be less than 10% in estuaries fed by rivers of iron content near the world average¹².

Phosphate in waters of the Yarra River estuary (Australia) shows large variations related to river flow (Fig. 1). At low flow (21 February 1977) filtered river water contained 185 $\mu\text{g P l}^{-1}$, and adjacent seawater in Port Phillip Bay¹³ contained 90 $\mu\text{g P l}^{-1}$, with a major input of phosphate part way along the estuary. After a period of high flow (24 June 1977) phosphate in the filtered river water was 32 $\mu\text{g P l}^{-1}$, with much of the phosphate in the estuary then being supplied from the seawater. This variability of phosphate distribution in the Yarra estuary made interpretation in terms of phosphate behaviour difficult, and a series of laboratory studies were undertaken to elucidate the effects of some of the non-biological factors.

Reactions in estuaries are strongly influenced by interactions of dissolved species with surfaces of suspended particles, and by aggregation of fine particles as salinity increases. Both types of interaction have the effect of converting 'dissolved' species into 'particulate' form, where the differentiation is by whether or not the species passes through a chosen filter. The ionic strength and pH of estuarine waters increase with increasing salinity, and a

Table 1 Starting solutions used in estuarine mixing experiments

No.	Humic acid (mg l^{-1})	Iron ($\mu\text{g Fe l}^{-1}$)	Phosphate ($\mu\text{g P l}^{-1}$)
1	0	0	185
2	9.0	0	184
3	1.2	100	160
4 (a)	0	750	124
5 (b)	0	750	42
6 (c)	9.0	750	174
7 (d)	0	750	144

Each solution initially contained 200 $\mu\text{g P l}^{-1}$ added as KH_2PO_4 . The results show the concentration of phosphate remaining in solutions that had been aged overnight, then filtered. a, P added after Fe. b, P added before Fe. c, P added after humic acid and Fe. d, Fe added 5 days before P. Results of adding seawater to unfiltered solutions a, b and c are shown in Fig. 3.

complex sequence of reactions ensues. For this reason we designed a laboratory system to simulate the progressive mixing of river water and seawater, as it occurs in an estuary, rather than using the alternative of batch mixing. The Yarra estuary is stratified¹⁴ with mixing occurring at different rates in the horizontal and vertical directions. A time of 1–2 h for doubling of the salinity was estimated for the horizontal direction, and this was later confirmed by J. Hinwood (personal communication). For estuaries that are much larger, especially if they are also well mixed, the half life for salinity change may be longer by orders of magnitude.

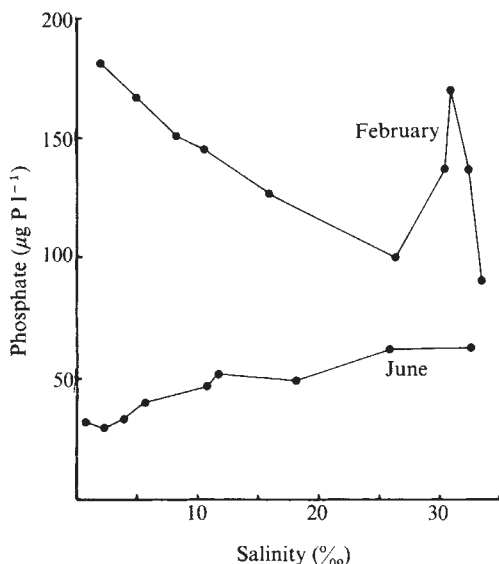


Fig. 1 Variation of dissolved phosphate with salinity in waters of the Yarra River estuary on two occasions in 1977.

In our laboratory model, seawater was pumped at a steady rate into a vessel containing river water, and the mixture pumped out at the same rate to maintain a constant volume. The solution was held at $15 \pm 0.1^\circ\text{C}$, and stirred at a constant rate to ensure rapid mixing and maintain particulate matter in suspension. The salinity approached that of the seawater exponentially, with a half life for salinity change governed by the rate of flow of the peristaltic pump used and the initial volume of river water. Adding seawater at 3.4 ml min^{-1} to 500 ml of river water gave a half life for salinity of about 90 min. Samples of the outflow were collected for analysis over a period of 5 h. Phosphate was determined colorimetrically¹⁵ on 10-ml samples collected in a graduated syringe and immediately filtered through $0.2\text{-}\mu\text{m}$ pore size Nuclepore filters. All acid-soluble phosphates, including dissolved and colloidal forms, would be included using this method. Iron was determined colorimetrically using ferrozine¹⁶ on filtered samples buffered at pH 5. Humic acid was determined from absorbance at 365 nm, calibrated using humic acid extracted from a large volume of Yarra river water. Salinity was determined to $\pm 0.1\%$ from refractive index.

In a laboratory experiment, estuarine mixing was simulated by adding seawater to unfiltered Yarra river water. The seawater from Bass Strait ($S = 35.0\%$, $\text{pH } 8.09$) contained $4 \mu\text{g P l}^{-1}$ which was unchanged by filtration. River water collected above seawater influence, contained 26 mg l^{-1} suspended matter and 1.9 mg l^{-1} humic acid and was $\text{pH } 7.25$. The filtered river water contained $99 \mu\text{g Fe l}^{-1}$ and $124 \mu\text{g P l}^{-1}$, compared with $1,800 \mu\text{g Fe l}^{-1}$ and $214 \mu\text{g P l}^{-1}$ in the acidified unfiltered water. Thus in the river water sample used, 94.5% of the iron and 42% of the phosphate was associated with the particulate matter. Behaviour of the three components measured during

mixing was non-conservative, with iron removal 70% (similar to that reported for other rivers^{12,17,18}), humic acid removal 23%, and phosphate removal 6%. The salinity of maximum removal was different for each component (Fig. 2).

A separate series of experiments using synthetic starting solutions allowed the effects of iron and humic acid to be differentiated. When FeCl_3 was added to water, the level of Fe detectable after filtration fell very rapidly to less than $5 \mu\text{g l}^{-1}$. Iron was stabilized in 'solution' by adding humic acid¹⁹. We found that 1.2 mg of humic acid per $100 \mu\text{g}$ of Fe was effective, using a filtered solution of 1.2 mg ml^{-1} Aldrich commercial humic acid in 0.1 M tris(hydroxymethyl)methylamine. The starting solutions listed in Table 1 were allowed to stabilize overnight before use, and the amount of phosphate in a small sample of filtrate was determined immediately before the start of each mixing experiment. The addition of iron after the phosphate caused much greater phosphate removal than occurred when the iron was added before the phosphate. When seawater was added to the unfiltered starting solutions in the estuarine mixing model, dissolved phosphate was essentially conservative in all cases except in the presence of high levels of iron and humic acid. Starting with 9 mg HA , 750 mg Fe and $200 \mu\text{g P l}^{-1}$, a maximum of 44% of the phosphate was removed from solution (Fig. 3).

The lack of interaction of iron with humic acid is expected as both are anionic. The indifference of iron to dissolved phosphate during estuarine mixing is related to the low concentration of iron in true solution in ionic form, and the chemical stability of hydrated iron oxide colloidal particles after they are formed. When associated with humic acids, iron is present both weakly bound on external surfaces, and as hydrated oxides²⁰. The

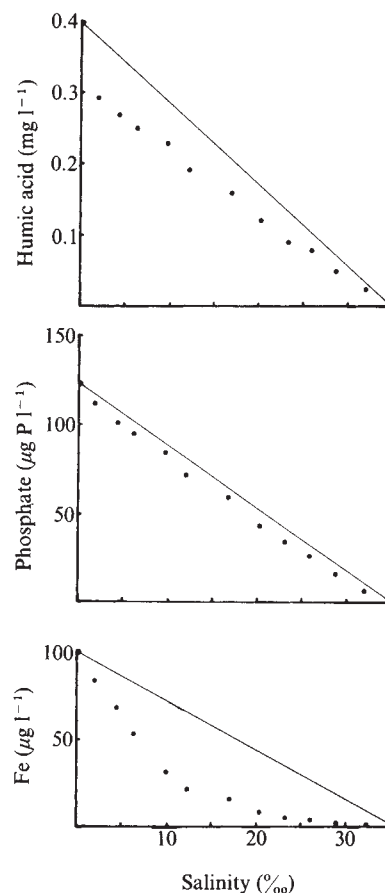


Fig. 2 Changes in concentration of filterable humic acid, phosphate and iron, when seawater was added to unfiltered Yarra River water in a laboratory model system.

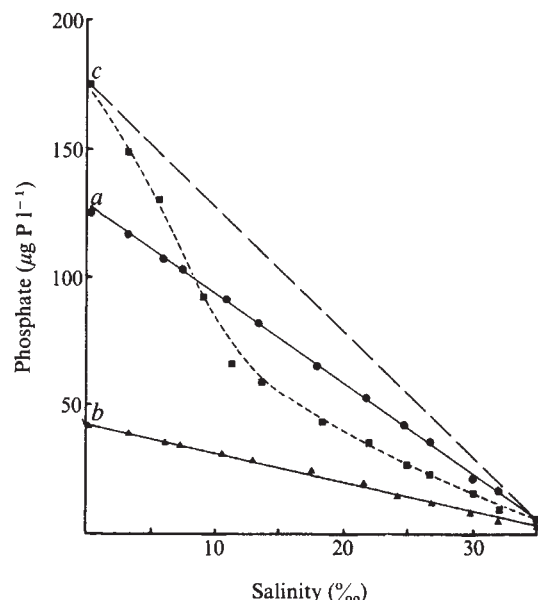


Fig. 3 Concentrations of filterable phosphate observed during addition of seawater to different synthetic solutions in a laboratory model system. Initial solutions were prepared as follows: a, phosphate added after iron; b, phosphate added before iron; c, phosphate added after humic acid and iron. The initial solutions were stirred overnight then used unfiltered.

weakly bound iron seems able to interact with dissolved phosphate. The presence of cations of Na, K, Ca and Mg, which are able to complex phosphate, is expected to increase the stability of dissolved phosphate in real river waters compared with the synthetic starting solutions²¹.

A consequence of the observed behaviour of phosphate is that the proportion of phosphate removed from solution in river water, before it enters the sea, will be largely controlled by the sequence and concentrations of the additions of iron, humic acid and phosphate to the river. In rivers exceptionally high in iron and humic acid, up to about half of the dissolved phosphate may be removed by inorganic processes in the estuary. For rivers near the world average concentration of iron¹², dissolved phosphate is likely to show conservative chemical behaviour in the estuary, or removal to only a small degree.

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Changes in spruce composition following burial in lake sediments for 10,000 yr

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Organic matter in lake sediments is a mixture of biological materials from a number of modern sources plus variable amounts of recycled detrital material. Hence, it is usually difficult to distinguish input variations over time from the diagenesis of organic matter. Comparison of old material of known biological origin with younger material helps resolve this problem. We have compared the compositions of wood and needles from a modern white spruce (*Picea glauca*) with those from a white spruce buried for 10,000 yr in a lake bottom. Although the tissues are structurally well preserved, some chemical changes are evident in the old samples. Total fatty acid concentrations decrease by over 90%. Sterol and hydrocarbon concentrations are similar in the modern and 10,000-yr-old wood, but the concentration of sterols is lower in the old needles. Cellulose components in the wood have decreased relative to lignin components, although both types of materials remain in high concentration in comparison to other organic components.

The types of chemical alterations which biological materials experience during early diagenesis in geological settings are important to biogeochemistry. A particularly interesting approach which has been used to describe some of these changes has involved chemical analysis of fossil materials, including bones¹, hair², corals³, and marine plankton^{4,5}. In addition, fossilized fruit⁶, leaves⁷ and wood^{8,9} have been studied.

We collected samples of wood and needles from a living white spruce (*P. glauca*) at The University of Michigan Matthaei Botanical Gardens near Ann Arbor. Similar samples were obtained from a tree which had been drowned and buried still erect in a lake bottom 10,000 yr ago. This lake was formed by glacial damming near present-day Marquette, Michigan, and was then quickly filled with sediment¹⁰. Lipid components of wood and needle samples were extracted in a Soxhlet apparatus with benzene-methanol for 48 h and by alkaline hydrolysis of the extracted samples in methanolic 0.5M KOH in benzene. The two extracts are analogous to the unbound and bound lipids released from sediments by a similar treatment¹¹. Each extract was fractionated by thin-layer and column chromatography into fatty acid, sterol, and aliphatic hydrocarbon fractions which were analysed by gas chromatography. Cellulose and lignin components of the modern and ancient wood samples were isolated by hydrolysis with 5M NaOH at 170°C for 12 h followed by extraction of the degradation products with ethyl acetate. These were analysed as their trimethylsilyl derivatives by GC and GC-MS.

As shown in Table 1, the concentrations of all of the needle components and some of the wood components are lower in the 10,000-yr-old samples than in the modern ones. If the biochemical processes of the white spruce have not changed over the past 10,000 yr, then these differences are due to alterations which have occurred in the older samples during burial in the water-saturated sediment.

Fatty acid concentrations show the greatest differences, and these reflect degradation of acids originally present in the wood

and needles. The unbound fractions have greater decreases in concentration than do the bound fractions. Because the unbound acids are extracted readily with solvent, it is probable they exist in forms that are vulnerable to both solution and microbial attack in an aqueous environment such as a lake sediment. There are some differences between the distributions in the wood and needle samples. For example, *n*-tetradecanoic acid is abundant in both the modern and old needles, but not in either wood sample. Also, *n*-docosanoic and *n*-tetracosanoic acids are important components of the unbound acids in the needles, but not in the wood. Another major difference is that the bound acid fraction is an order of magnitude more abundant in the needle samples.

Some of the fatty acid changes are shown in Fig. 1. These compositions are of wood samples; comparable changes were observed in the needles. Relative proportions of unsaturated acids are less in the older wood, whereas the contributions of *anteiso* methyl-branched acids indicative of microbial processes increases. Longer-chain *n*-alkanoic acids seem to be degraded less in the bound acids. Similar differences have been found in fatty acids extracted from modern and 12,000-yr-old Lake Huron sediments¹² and evidently are due to diagenesis rather than changes in input.

Unbound sterol concentrations of the needles indicate a time-related loss of the same magnitude as found in the fatty acids. However, the bound sterol concentration increases from essentially zero to $55 \mu\text{g g}^{-1}$, suggesting conversion of some unbound sterols to bound forms. A similar conversion has been reported in young sediments^{13,14} and also seems to occur in the wood samples. In the wood, this is accompanied by a small increase in the concentration of unbound sterols. Because diagenetic formation of sterols from other wood components is unlikely, the apparent increase in concentration suggests preferential losses of proteinaceous and saccharide materials, with the result that sterols comprise a larger portion of the old wood. Sterols are evidently only mildly reactive in wood. This also appears to be true in sediments¹³⁻¹⁵.

Although at lower concentrations than the sterols, aliphatic hydrocarbons behave similarly. The concentration of unbound hydrocarbons is lower in the old needles than in the new, but higher in the old wood than in the modern sample. Unlike the sterols, no conversion of hydrocarbons from the unbound form to the bound form is evident. In view of the lack of reactive functional groups in saturated hydrocarbons, this is not surprising. Normal alkanes from C_{16} to C_{37} are present in these samples, and no major compositional changes are found between the 10,000-yr-old and modern samples.

The changes in the lipid components of the needles suggest that the unbound lipids are easily removed or degraded over

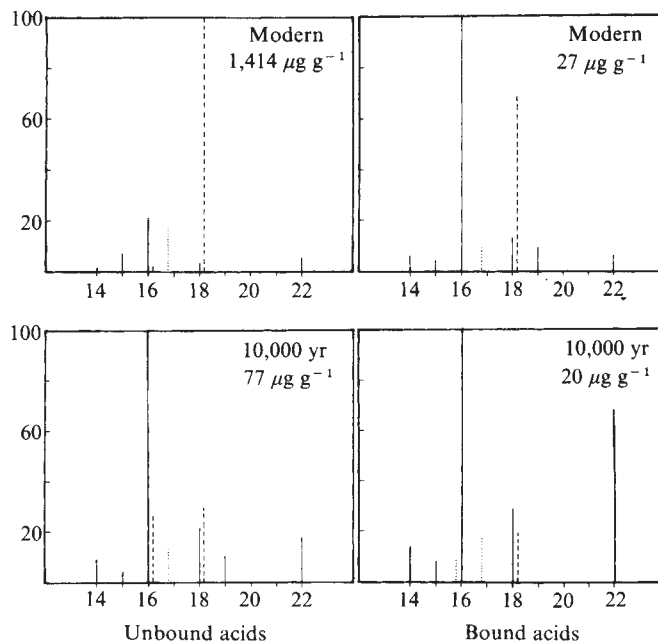


Fig. 1 Comparison of fatty acids extracted from modern and 10,000-yr-old spruce wood. Unbound acids released by Soxhlet extraction; bound acids by alkaline hydrolysis of extracted wood. Compositions are relative to major acid component (100%). Acids are displayed by number of carbon atoms. Solid lines represent *n*-alkanoic acids; dashed lines *n*-alkenoic acids; dotted lines *anteiso* acids.

geologically short periods of time in sediments. Much of this material may exist as a waxy coating on the needles which is exposed to microbial degradation, chemical weathering and dissolution. Unbound lipids in wood are internal structural components and therefore not as available to similar attack as are the needle-coating lipids. Even so, wood fatty acids are destroyed or converted to non-acid forms relatively quickly. Evidently, preservation of fatty acids in sediments requires special circumstances, such as anoxic conditions and rapid burial.

The cellulose plus lignin component of the old wood is at a higher concentration than in the modern wood. As suggested in the case of wood sterols and hydrocarbons, this indicates less alteration of these materials relative to other, more labile components. Lactic acid is the most abundant compound found in these samples. Oxalic and succinic acids are present as hydrolysis products of cellulose¹⁶. Their proportions relative to each other are the same in both samples. Vanillic acid and ferulic acid are also found and are derived from lignin^{16,17}. The contribution of cellulose components, represented by oxalic and succinic acids, decreases relative to that of lignin components, represented by vanillic and ferulic acids, in the old wood. The ratio of these components changes from 2.2 in the modern wood to 1.2 in the old, indicating a selective loss of cellulose compared to lignin over this 10,000-yr period of burial.

These data give relative rates of degradation of some types of biological compounds buried in lake sediments for short periods of geological time. In woody tissue, the order of alteration is fatty acids > sterols > aliphatic hydrocarbons > cellulose > lignin. In spruce needles, the order is fatty acids = sterols > aliphatic hydrocarbons. These relative rates can be useful for understanding the cycling of organic matter in young subaqueous sediments. Furthermore, this study describes some woody tissue changes which occur during the early stages of coalification.

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Table 1 Extractable components of modern and 10,000-year-old wood and needles from the white spruce *Picea glauca*

Component	Wood			Needles		
	Modern	Old	Δ	Modern	Old	Δ
Fatty acids						
unbound	1,414	77	-1,337	2,785	114	-2,671
bound	27	20	-7	362	237	-125
total	1,441	97	-1,344	3,147	351	-2,796
Sterols						
unbound	311	339	+28	2,159	107	-2,052
bound	2	11	+9	0	55	+55
total	313	350	+37	2,159	162	-1,997
Hydrocarbons						
unbound	27	36	+9	146	54	-92
bound	1	1	0	1	1	0
total	28	37	+9	147	55	-92
Cellulose plus lignin	157	192	+35			

Concentrations of fatty acids, sterols and hydrocarbons are given in $\mu\text{g per g dry wood}$; cellulose plus lignin components are in mg per g . Δ , Difference between modern and old concentrations.

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Evidence for diploidy and mating in trypanosomes

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The question of whether any sexual process takes place at some stage of the life cycle of the trypanosome has often been raised^{1,2} and crosses between different drug-resistant strains^{3,4} have provided no convincing answer. Multinucleate forms of trypanosomes have been observed by electron microscopy^{5,6} but their significance and origin remain obscure. As part of a study aimed at examining the speciation and genetics of the *Trypanosoma brucei* complex of trypanosomes, a series of isolates from a population of *T. b. brucei* have been screened for electrophoretic variation in 19 enzymes. The results of this survey, reported here, provide strong evidence that trypanosomes are diploid and undergo random mating and recombination.

The population used consisted of 17 stocks of *T. b. brucei*, collected in 1969-70 from Lugala, Uganda, by members of the East African Trypanosomiasis Research Organisation (EATRO). Fifteen of the stocks were obtained by injection of infected tsetse salivary glands into mice and subsequent storage of infected blood in liquid nitrogen. The remaining two stocks were obtained by injection of infected blood from wild game into mice, followed by storage in liquid nitrogen. Samples of these stocks were transferred to the Centre for Tropical Veterinary Medicine in Edinburgh. This material was made available by Dr A. Gray and preparations of trypanosomes were obtained by infecting laboratory mice with these stocks, followed by DEAE-cellulose chromatography⁷ of infected blood. Extracts from such purified preparations were then subjected to starch gel electrophoresis and the gels specifically stained for the 19 enzymes listed in Table 1.

Electrophoretic variation was observed in eight of the enzymes, which can be subdivided on the basis of the type of variant banding pattern obtained on gels (Table 1). In type I, with, for example, isocitrate dehydrogenase, some stocks produce a single band of enzyme activity at one position on the gel (1CD-1), others produce a single band at a second position (1CD-2), and a third group of stocks produce three bands, two of which correspond to 1CD-1 and 1CD-2, respectively, the third (of double intensity) lying midway between 1CD-1 and 1CD-2 (Fig. 1a). In type II, with, for example, alkaline phosphatase, individual stocks may produce single bands at one of two alternative positions (AP-1 or AP-2), whereas other stocks produce two bands at positions corresponding to AP1-1 and

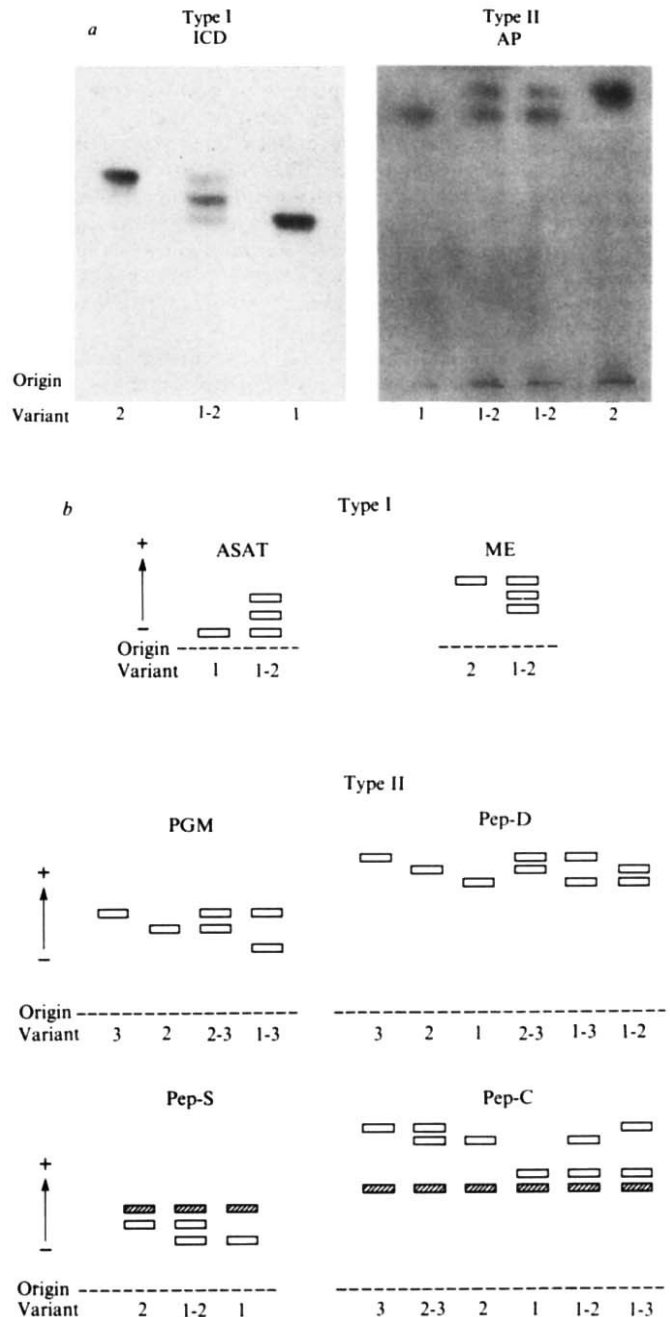


Fig. 1 Electrophoretic enzyme variation in isolates of *T. b. brucei*. *a*, Photographs of starch gel electrophoresis slabs stained for isocitrate dehydrogenase (ICD) and alkaline phosphatase (AP). Samples were run in 9% hydrolysed starch gels in 0.026 M Tris, 0.0033 M citric acid, pH 8.6, at 270 V for 3 h. The electrode buffer was 0.66 M Tris, 0.083 M citric acid, pH 8.6, and the enzyme activities were stained using published methods¹⁶. *b*, Diagrams of starch gel electrophoresis slabs stained for aspartate amino transferase (ASAT), malic enzyme (ME), phosphoglucosyltransferase (PGM), peptidase-S (Pep-S), peptidase-C (Pep-C) and peptidase-D (Pep-D). The peptidases are designated on the basis of their di- and tripeptide specificities using the nomenclature described by Harris and Hopkinson¹⁶; the cross-hatched bands shown on Pep-S and Pep-C gels represent the two invariant peptidases (Pep-A and Pep-B, see Table 1) which overlap in their substrate specificities. Details of the electrophoretic conditions and activity stains will be published elsewhere. The variant bands observed are designated by numbers in order of increasing mobility.

AP-2 (Fig. 1a). Of the eight polymorphic enzymes found, three are of type I and five of type II (Table 1). Furthermore, four of the type II enzymes show more than two possible positions at which the bands may appear. The variants observed for the eight polymorphic enzymes are shown in Fig. 1a and b, together with the nomenclature used for their designation. With all these enzymes multiple-banded types were observed, triple banded in the case of type I and double banded in the case of type II.

Table 1 Electrophoretic enzyme variation in *T.b. brucei*

Enzyme	Abbreviation	Mono-morphic	Poly-morphic	Type	Alleles
Threonine dehydrogenase	TDH	+	—	—	—
6-phosphogluconate dehydrogenase	6PGD	+	—	—	—
Glucose-6-phosphate dehydrogenase	G6PD	+	—	—	—
Mannose-phosphate isomerase	MPI	+	—	—	—
Glucose phosphate isomerase	GPI	+	—	—	—
Malate dehydrogenase	MD	+	—	—	—
Phosphoglyceromutase	PGlyM	+	—	—	—
Heptanoyl esterase	Hest	+	—	—	—
Acetyl esterase	Acest	+	—	—	—
Peptidase-B (prolyl-leucine)	Pep-B	+	—	—	—
Peptidase-A (valyl-leucine)	Pep-A	+	—	—	—
Isocitrate dehydrogenase	ICD	—	+	I	2
Alanine aspartate amino transferase	ASAT	—	+	I	2
Malic enzyme	ME	—	+	I	2
Phosphoglucomutase	PGM	—	+	II	3
Alkaline phosphatase	AP	—	+	II	2
Peptidase-S (tyrosyl-tyrosine)	Pep-S	—	+	II	2
Peptidase-C (leucyl-alanine)	Pep-C	—	+	II	3
Peptidase-D (phenyl-alanyl-proline)	Pep-D	—	+	II	3

Summary of the results obtained by screening 17 stocks of *T.b. brucei* for electrophoretic enzyme variation. Type I and type II refer to the type of multiple banding observed (see text). Alleles refers to the presumed number of alleles determining each enzyme.

Table 2 Numbers of stocks of *T.b. brucei* showing different enzyme genotypes

Enzyme	Geno-type	Observed	(Expected)	Enzyme	Geno-type	Observed	(Expected)
ICD	1	3	(3.3)	Pep-S	1	6	(5.27)
	1-2	9	(8.38)		1-2	7	(8.33)
	2	5	(5.34)		2	4	(3.23)
PGM	1	1	(0.06)	Pep-C	1	3	(1.77)
	1-2	0	(0.36)		1-2	3	(4.18)
	2	1	(0.53)		2	3	(2.47)
	2-3	4	(4.57)		2-3	4	(3.81)
	3	10	(9.95)		3	2	(1.46)
ASAT	3-1	2	(1.58)		3-1	2	(3.23)
	1	9	(9.9)	Pep-D	1	0	(0.02)
	1-2	8	(6.1)		1-2	3	(1.7)
	2	0	(1.0)		2	4	(2.86)
ME	1	0	(0.39)		2-3	3	(6.46)
	1-2	5	(4.34)		3	6	(7.99)
	2	12	(12.29)		3-1	1	(1.92)
				AP	1	2	(3.7)
					1-2	12	(8.3)
					2	3	(4.7)

Genotypes are designated as described in Fig. 1a,b. Expected genotype numbers were calculated from the observed allele frequencies assuming that the population was in Hardy-Weinberg equilibrium.

Hybrid banding patterns such as those seen in types I and II enzymes would be expected in a diploid organism in which some individuals are heterozygous for allelic variants of dimeric (in the case of type I) and monomeric (in the case of type II) enzymes. Similar patterns of multiple banding have been shown for many enzymes in, for example, humans⁸, *Drosophila*⁹ and mice¹⁰, and have been demonstrated to be due to individuals heterozygous for the different alleles. Thus, the observation of both single-banded and hybrid-banded electrophoretic enzyme patterns suggests, by analogy with other organisms, that trypanosomes are diploid and that mating has taken place to generate the 'heterozygous' patterns. Godfrey and co-workers have reported variation in enzyme electrophoretic mobility in several different species and subspecies of trypanosomes¹¹⁻¹³ and with five of the enzymes 'hybrid' band patterns have been

reported^{14,15} in stocks of the *T. brucei* complex. These results would also support the interpretation of the observations presented here.

An alternative explanation, in the case of the type II enzymes, might be based on the assumption that the two-banded patterns are not due to heterozygotes, but to mixtures of two different clones. It is unlikely, however, that the stocks used in this study were, in fact, such mixtures. Four stocks exhibiting multiple band patterns were cloned by infecting mice with single trypanosomes and the resulting clones examined by starch gel electrophoresis. All four cloned stocks again showed multiple-banded patterns for all eight polymorphic enzymes. Thus, none of the multiple-banded patterns shown by these stocks could have been due to mixtures of clones. Moreover, in the case of the type I enzymes which show three bands of activity, the band of intermediate mobility was only observed in conjunction with the two other bands; nor were there any stocks showing mixtures of the latter without the intermediate band. It is, therefore, assumed that most of the stocks showing multiple-banded patterns were heterozygotes. Based on this assumption, the allele frequencies for these enzyme variants and from them the expected genotype frequencies can be calculated, assuming that the population is in Hardy-Weinberg equilibrium. The observed and predicted genotype proportions are presented in Table 2 for the eight enzymes studied. Clearly, expected and observed proportions are in good agreement and the goodness of fit has been examined using the χ^2 test. The values obtained show that the observed proportions of genotypes do not differ significantly at the 5% level from those predicted by the Hardy-Weinberg equilibrium. This finding, together with the similarity of the banding patterns to those observed with known heterozygotes in other organisms and their occurrence in all variant enzymes examined, provides strong evidence that these trypanosome strains are diploid and undergo random mating.

The data also provide evidence for the occurrence of genetic recombination between genes at different enzyme-determining loci. Classification of the stocks with regard to variants of any two enzymes simultaneously shows (Table 3) that 50 out of a possible total of 90 recombinant types occur, even with the small number of stocks studied. As the number of isolates is small, comparisons have only been made between pairs of enzymes showing two alleles. This strongly suggests that recombination has occurred between the loci specifying these enzymes, as the frequency of possible combinations of variants generated by

Table 3 Combinations of variants of AP, ASAT, ICD, ME and PEP-S

	AP			ASAT			ICD			ME			PEP-S		
	1	1-2	2	1	1-2	2	1	1-2	2	1	1-2	2	1	1-2	2
AP															
1															
1-2															
2															
ASAT															
1		1	7	1											
1-2		1	6	1											
2		0	0	0											
ICD															
1		0	3	0	3	0	0								
1-2		2	7	0	4	5	0								
2		0	3	2	2	3	0								
ME															
1		0	0	0	0	0	0	0	0						
1-2		0	5	0	3	2	0	3	0	2					
2		2	8	2	6	6	0	0	9	3					
PEP-S															
1		1	5	0	2	4	0	0	5	1	0	0	6		
1-2		1	4	2	3	4	0	1	2	4	0	3	4		
2		0	4	0	4	0	0	2	2	0	0	2	2		

The numbers of stocks showing the various possible pairwise combinations of enzyme variants. See Table 1 for abbreviations used. A total of 17 stocks was examined.

mutation in the absence of recombination would be expected to be very low.

The present findings provide strong evidence for diploidy, random mating and recombination between different loci in this population of trypanosomes. Conclusive proof that these organisms are diploid and undergo mating would require the demonstration of heterozygotes produced by hybridization of cells showing different variants of the same enzyme. Experiments are under way (using a range of genetic markers) to demonstrate such hybridization.

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Changing proteins on the surface of a parasitic nematode

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Most of the organisms of the phylum Nematoda are free living, but some are animal or plant parasites of major importance to man. During their life cycle all nematodes undergo a series of moults in which they shed an external cuticle, consisting of an outermost membrane-like layer of unknown composition and a series of fibrillar layers similar to collagens^{1–4}. Because of this structure, the cuticle has been viewed as an acellular exoskeleton¹ with rather inert molecular components. However, observations have shown that it contains enzymes and sometimes haemoglobin^{3,4}, and that nutrients are absorbed through it in the infective larvae and adult stages of *Brugia pahangi*⁵. It is bound by complement and antibody, resulting in the adherence of leukocytes⁶, and antibody-dependent cell-mediated reactions damage the cuticle of newborn larvae of *Trichinella spiralis*^{7–9} and the microfilariae of *Dipetalonema viteae* and *Litomosoides carinii*^{10,11}. We report here that the surface of the cuticle of the parasitic nematode *Trichinella spiralis* expresses protein molecules which change qualitatively following the moulting process, and quantitatively during growth of the worms within one stage. Also, surface proteins are released *in vitro* at a rate which depends on the conditions of culture of the worms.

If the cuticle is functional it should have a more dynamic molecular structure than has so far been described. As parasitic nematodes are exposed to very different host environments during their life cycle, we chose to study surface components of one of them—*T. spiralis*.

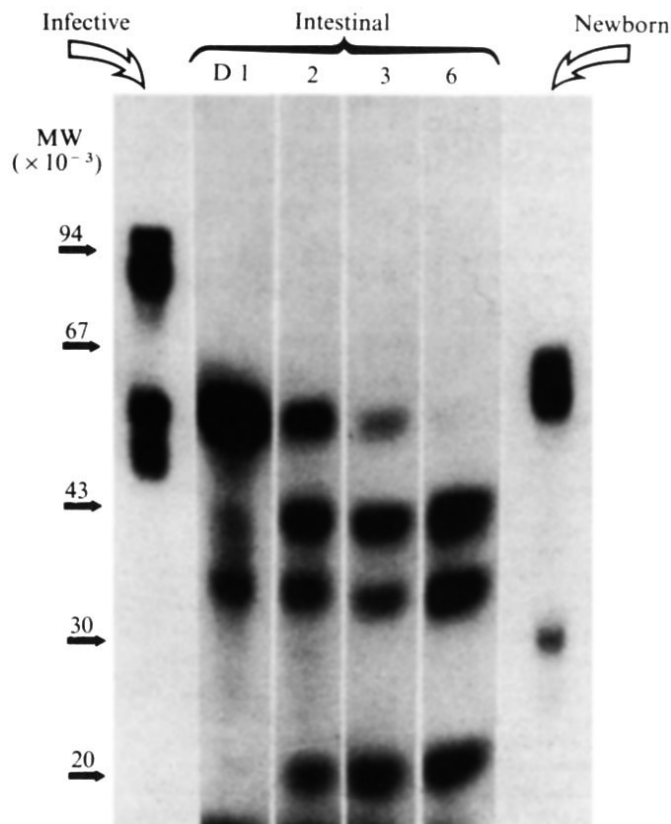


Fig. 1 Radiolabelled surface proteins of the three stages of *T. spiralis*: infective larvae, intestinal worms (removed from small intestines at days 1, 2, 3 and 6 after oral infection), and newborn larvae. About 10^4 infective larvae, 10^4 intestinal worms and 10^5 newborn larvae were washed 10 times in phosphate-buffered saline (PBS) by low-speed centrifugation. To 0.1–0.5 ml of PBS containing the worms and 0.1–1 mCi Na^{125}I , chloramine T was added to a final concentration of 0.050 mg ml^{-1} . The mixture was left to react at room temperature for 3 min after which tyrosine was added to react with the uncombined iodine. As judged by mobility, the worms were 90–100% viable after labelling. The worms were washed repeatedly in PBS and then lysed in Tris-P buffer (10 mM Tris-HCl, pH 8.3, $50 \mu\text{g ml}^{-1}$ L-1-tosylamide-2-phenylethylchloromethyl ketone, $25 \mu\text{g ml}^{-1}$ N- α -p-tosyl-L-lysine-chloromethyl ketone-HCl, 1 mM phenylmethyl sulphonyl fluoride and 2% Na deoxycholate). Intestinal worms and infective larvae were disrupted by homogenization in a glass-glass homogenizer, whereas sonication was used with newborn larvae. The lysates were centrifuged for 30 min at 11,000g and the supernatant, containing 90–95% of the total radioactivity (70–80% acid precipitable) was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Slab gels containing 7.5% acrylamide were used. The samples were boiled for 5 min in a buffer solution containing 8.3 mg ml^{-1} β -mercaptoethanol and 17.0 mg ml^{-1} SDS. The radiolabelled material has been shown to be located on the worms surface by: (1) Autoradiographs of sections of radiolabelled worms. (2) Absorption of antibodies to the labelled proteins by whole intact worms. (3) Alteration of the rate of turnover of the radiolabelled proteins by neutrophil attachment to the worms' surface (this letter). The SDS-PAGE pattern of the infective larvae was neither altered when the worms were radiolabelled 72 h after the pepsin digestion of rat muscle, nor when radiolabelled worms were incubated with pepsin in similar conditions to the ones used in the digestion process, before homogenization.

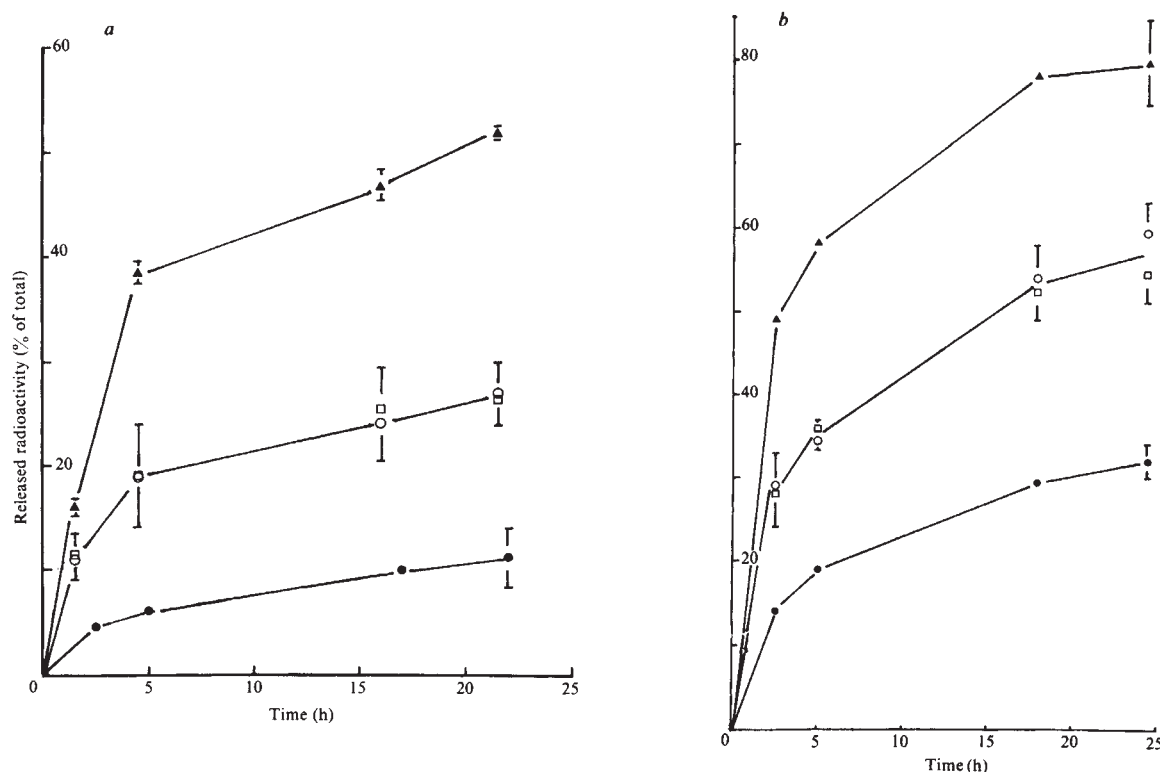


Fig. 2 Release of radiolabelled proteins from the surface of *a*, infective larvae and *b*, intestinal worms (day 2) cultured in RPMI (●), normal rat serum (□), immune rat sera (○), in RPMI and immune rat sera plus rat neutrophils (▲). RPMI 1640 (Gibco) was buffered with NaHCO_3 and 20 mM HEPES to pH 7.2, and 1×10^5 units ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin and 1% glucose were added. About 10 living radiolabelled worms were incubated in 0.4 ml of the above media at 37°C . Aliquots (5 μl) were taken at intervals and from the measured radioactivity the average released amount was calculated and expressed as per cent of the initial radioactivity of the worms. All data points were determined in triplicate. Error bars represent maximum dispersion of average data points corresponding to duplicate experiments run on two different occasions. Normal rat sera were collected from Sprague-Dawley rats and kept at -20°C until used. Immune rat sera were taken from Sprague-Dawley rats 21 days after oral infection with 4,000 infective larvae of *T. spiralis* and kept at -20°C until used. Neutrophils were obtained by peritoneal lavage of Sprague-Dawley rats 4 h after they had been given a peritoneal injection of 10 ml of 1% glycogen (Sigma) in PBS. This yielded about 80% neutrophils. About 2×10^7 cells were used in each experiment.

The life cycle of *T. spiralis* can be reproduced in the laboratory using rodent hosts¹². The infective larvae, which are found in the striated muscle of vertebrate hosts, can be recovered by enzymatic digestion of the muscular tissue¹². When such muscle is ingested by another animal, the infective larvae undergo a very rapid series of moults that are completed within 24 h, and the worms become adapted to the small intestine of the host. The intestinal stage worms grow and become sexually mature during the first 5 days after oral infection, and can be recovered from the intestine between days 1 and 6 (ref. 12). The newborn larvae, which are shed from the mature female worms after mating can be recovered by maintaining the 6-day-old intestinal stages *in vitro* overnight¹². In *in vivo* conditions the newborn larvae make their way through the wall of the small intestine and migrate to striated muscle cells of the new host, thus completing the life cycle.

Infective larvae, intestinal worms and newborn larvae were surface-labelled with ^{125}I -labelled iodine, as described in Fig. 1 legend. The radiolabelled surface components of each stage, including four different phases in the maturation of the intestinal stage, were analysed by electrophoresis on SDS-polyacrylamide slab gels. An autoradiograph of such a slab gel is shown in Fig. 1. All samples are reduced with β -mercaptoethanol.

The infective larvae expressed four main surface proteins of apparent molecular weights (MWs) 47,000, 55,000, 90,000 and 105,000. The intestinal stage presented a qualitatively different pattern of surface proteins. At day 1 after oral infection extracts of labelled worms gave one predominant band of MW 56,000 on

the gels. However, unlike the molecule of MW 55,000 seen on infective larvae, this component did not bind to lentil lectin (data not shown). A second fainter band ran at MW $\sim 33,000$ (Fig. 1, D1) and traces of radiolabelled protein could be detected at MWs $\sim 40,000$ and 20,000 in extracts of 1-day-old intestinal worms.

During maturation of the intestinal worms—days 1 to 6 after infection—an inversion in the relative intensity of the bands took place. Thus, the intensity of the 56,000 MW band decreased, and the 40,000- and 20,000-MW bands became gradually more intense. A slight intensification of the 33,000-MW band could also be seen.

The surface protein pattern of the newborn larvae was qualitatively different compared with the other two stages. There were two bands running at approximate MWs of 58,000 and 64,000, and a pair of comparatively faint bands of MWs 28,000 and 30,000. Thus, whereas the surface proteins changed qualitatively in the transition between any pair of different stages, only quantitative changes took place when the intestinal worms passed through the different phases in their maturation.

Further evidence for a dynamic cuticle came from experiments which showed that the radiolabelled surface proteins of the infective larvae and the intestinal worms may be released by the worms *in vitro*. Worms of either stage were radiolabelled with ^{125}I , as described in Fig. 1 legend, and incubated at 37°C in four different culture conditions: RPMI, normal rat serum, immune rat serum and immune rat serum plus rat neutrophils. Samples of the culture fluid were taken at intervals and the

released radioactivity was expressed as a percentage of the total radioactivity present on the worm's surface at time zero. A plot of the released radioactivity against time for both the infective larvae and intestinal worms is shown in Fig. 2a and b respectively. Radiolabelled material was released in all four culture conditions. The rate of release was slowest in RPMI. In this medium the infective larvae released the 55,000-MW protein first and then the 105,000-MW component. The other two surface components were not released even after 48 h in culture. The intestinal worms released all four of their radiolabelled surface components in RPMI. The release of proteins was twice as fast when the worms were left in normal rat sera or sera from immune rats (previously infected with *T. spiralis*). This phenomenon cannot be explained in terms of different viability of the worms as they started to die only after 48 h in culture either in RPMI or in rat sera with or without antibody. Antibodies did not seem to affect the rate of protein release although they could be shown to bind to the radiolabelled proteins, in as much as these co-precipitated with immune rat sera and an anti-rat immunoglobulin antiserum.

A marked increase in the rate of release was achieved by co-culturing the worms with rat neutrophils (5×10^7 cells per ml) in the presence of immune rat sera (Fig. 2). Neutrophils adhered to the surface of both the infective larvae and the intestinal worms and began to dissociate from the worms' surface after 3–4 h in culture. Similarly, enhanced release of surface molecules was obtained when the labelled worms were cultured with normal serum and neutrophils (data not shown). These results indicate that the radiolabelled proteins are located on the surface of the cuticle, possibly on the outermost membrane-like layer, as neutrophils can be shown to remove this layer by about 4 h in culture (M. Jungery, personal communication). Furthermore, antibodies which precipitated the radiolabelled proteins also bound to intact worms, and autoradiographs of sections of radiolabelled worms showed that the label was on the cuticle and not on internal organs of the worm (M.P., R.M.E.P. and B.M.O., unpublished observations). The label was not concentrated in any particular region of the cuticle. However, release of protein material from the region of the bacillary pores cannot be ruled out nor confirmed with our present data.

In conclusion, our results indicate that the cuticle of *T. spiralis* is a dynamic organ, the surface of which shows dramatic changes at the molecular level during the moulting process and more gradual modifications during growth within one stage. The qualitative modifications of the surface proteins during a change in stage and antigenic evidence imply that these molecules are stage-specific. The surface proteins are strikingly few in number and are released into the medium when *T. spiralis* is maintained *in vitro*. This suggests that the secretions and excretions of nematodes include proteins shed from the cuticle.

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Sharing of an idiotypic marker by monoclonal antibodies specific for distinct regions of hen lysozyme

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Idiotypes have been defined serologically as variable-region markers present on unique subsets of antibody molecules. Most studies involving myeloma proteins and antibody responses in inbred mice have indicated that idiotype is closely related to antigenic specificity. However, indirect evidence^{1–7} suggests that idiotype can sometimes be dissociated from specificity. In the response to the small monomeric protein, hen egg-white lysozyme (HEL), previous results^{8,9} demonstrated that 70–95% of the anti-HEL antibody found in sera of primed and boosted mice is specific for a particular region of HEL and bears a predominant idiotype (IdX-HEL). In this report, hybridoma anti-HEL antibodies tested with well-characterized peptide fragments of HEL show that the IdX-HEL marker can be found on antibodies of completely distinct antigen specificities. Thus, a mode of recognition other than antigenic recognition probably plays a major role in the immune response to HEL.

Hybridoma anti-HEL antibodies were obtained by fusing HEL-primed A/J spleen cells with the nonsecreting BALB/c myeloma cell line, S194/5.XXO.BU.1 (Salk Institute). The growing hybrids were cloned by limiting dilution and monoclonality was confirmed by isoelectric focusing of several individual subclones, all of which for a particular hybridoma were indistinguishable with regard to spectrophototype. The activity of the monoclonal antibodies was determined in a solid-phase radioimmunoassay¹⁰ by direct binding to HEL and, in addition, this assay was used with peptide fragments that define discrete segments of the HEL molecule (N-C peptide amino acids 1–17, Cys 6–Cys 127, 120–129; mixed disulphide L_{II} peptide amino acids 13–105; and mixed disulphide L_{III} peptide amino acids 106–129) to assess specificity. The N-C peptide overlaps with L_{II} for a small sequence of five amino acids (13–17); L_{II} and L_{III} are non-overlapping peptides. Bovine ribonuclease, a protein of similar size and charge to HEL, was used to assess background levels of binding. As seen in Table 1, the anti-HEL hybridoma antibody, 6D7, recognizes the L_{II} but not the N-C peptide. Conversely, the hybridoma antibody, 5E4, recognizes HEL and the N-C peptide. In other experiments (not shown) the peptides

Table 1 Specificity of hybridoma antibodies 6D7 and 5E4

Hybridoma	Antigen on plate: radioactivity bound (c.p.m. $\times 10^{-3}$)			
	HEL	Ribonuclease	N-C	L _{II}
6D7	19.7	1.8	1.9	13.2
5E4	17.9	0.7	13.9	0.8

Specificity was assessed in a solid-phase radioimmunoassay in which the wells of microtitre plates (Cooke) were coated with 100 μ l of 0.5 mg ml⁻¹ antigen at 4 °C overnight. The plates were then flooded with a solution of 1% bovine serum albumin in phosphate-buffered saline (1% BSA-PBS) at room temperature for 1 h to saturate the protein-binding capacity of the wells. Next, the hybridoma supernatants (25 μ l) were added to the wells at room temperature for 2 h. Binding was assessed using 50 μ l of affinity-purified ¹²⁵I-goat anti-mouse Ig (10⁵ c.p.m. per well) at room temperature for 1 h. Between each specific addition, the plates were washed extensively with 1% BSA-PBS. 6D7 is an IgG2b κ monoclonal antibody and 5E4 is an IgG1 κ monoclonal antibody as determined by the solid-phase radioimmunoassay using rabbit anti-subclass antisera (provided by Drs J. Teale and N. Klinman, Scripps Clinic and Research Foundation) to assess binding to HEL. The results are expressed as c.p.m. bound per well.

Table 2 Competitive inhibition of the binding of hybridoma antibodies 6D7 and 5E4

Inhibitor	Amount of inhibitor (μ g)	Inhibition of 6D7 binding (%)		Inhibition of 5E4 binding (%)	
		HEL plate	L _{II} plate	HEL plate	N-C plate
HEL	0.001	16	24	5	0
	0.1	88	78	45	94
	10	92	88	94	92
Ribonuclease	10	0	0	0	0
L _{II}	10	58	90	0	0
N-C	10	0	0	33	72

Inhibition studies were performed using the solid phase radioimmunoassay and a dilution of hybridoma supernatant capable of 40–50% maximal binding to the insolubilized antigen. The supernatants (25 μ l) were preincubated with 25 μ l of 1% BSA-PBS to determine control binding or with 25 μ l soluble inhibitor at 37 °C for 30 min and then at 4 °C overnight. The mixtures were incubated at room temperature for 2 h in wells coated with the target antigen after which the wells were washed and incubated at room temperature for 1 h with 75 μ l of ¹²⁵I-goat anti-mouse Ig (10⁵ c.p.m. per well). As much as 1 μ g of L_{II} or N-C did not cause significant inhibition of binding of 6D7 or 5E4, respectively.

were coupled to keyhole limpet haemocyanin (KLH) to increase the sensitivity of detection and significant binding to L_{III}-KLH over background was observed with 5E4 but not 6D7. Thus, 5E4 appears to have specificity for a determinant in the C-terminal portion of HEL (amino acids 120–129) whereas 6D7 is specific for an epitope within the middle region of HEL (amino acids 17–105).

These non-overlapping specificities were confirmed by competitive inhibition of the radioimmunoassay in which the hybridoma antibodies were pre-incubated with the soluble peptides and then assayed for binding to insolubilized HEL and its fragments. As seen in Table 2, L_{II} but not N-C competed effectively for the binding of 6D7 to HEL (and L_{II}). On the other hand, N-C but not L_{II} inhibited the binding of 5E4 to HEL (and N-C). Thus, 5E4 and 6D7 recognize distinct epitopes on the HEL molecule. The need to use the peptides in large amounts relative to HEL for inhibition suggests that the antibodies have greater affinity for the native, conformationally restricted, HEL molecule than for the peptides.

The idiotype of the two monoclonal antibodies was investigated next. It has been found⁸ that anti-IdX-HEL antiserum appears to define an idiotype marker which resides close to the antigen-combining site and is present on the majority of secondary response anti-HEL antibody. Thus, anti-IdX-HEL antiserum, unlike polyvalent anti-Ig, interferes with the binding of HEL to its antibody in a standard Farr-type assay. The presence of IdX-HEL on 5E4 and 6D7 was assessed in a similar manner using a guinea pig anti-IdX-HEL antiserum raised against a third hybridoma anti-HEL, AD2. AD2, although not showing detectable binding to peptide fragments, does exhibit a relatively rare pattern of cross-reactions different from either 5E4 or 6D7 when tested with a panel of related lysozymes. The anti-IdX-HEL antiserum induced with AD2 appears to be similar to anti-idiotype raised against serum anti-HEL in that it interferes with the binding of HEL to most of the secondary anti-HEL serum antibody of B10.A mice. As shown previously⁹, such antibody is specific primarily for the N-C peptide. The anti-IdX-HEL antiserum against AD2 does not affect the binding of an essentially non-HEL cross-reactive lysozyme, human lysozyme, to its antibody (D. W. M., unpublished observations). As seen in Fig. 1, the recognition of HEL by 5E4 or 6D7 was in both cases almost completely inhibited by the anti-IdX-HEL antiserum, presumably by interaction with variable region residues not directly within, but close to, the antigen-combining site. In contrast, an anti-HEL hybridoma antibody, 2G3, which appears to lack the IdX-HEL marker, was almost

unaffected by anti-idiotype treatment. We have also found that the individual hybridoma product, 6D7 (as well as 5E4), can remove all the activity from an anti-idiotype antiserum raised against conventional serum anti-HEL antibody¹¹. Thus, two monoclonal antibodies that clearly differ in peptide specificity for a multideterminant antigen can both bear the IdX-HEL marker.

Earlier studies from our laboratory demonstrated that the majority of serum antibody produced in secondary responses of mice shared two properties: specificity for the N-C portion of HEL and presence of a common idiotype, IdX-HEL^{8,9}. The small amount of L_{II}-specific antibody that was detectable appeared to lack the IdX-HEL marker. Nevertheless, using hybridoma technology we have been able to detect L_{II}-specific, idiotype-positive antibodies.

In previous work^{11,12} we found that anti-HEL antibodies displaying the IdX-HEL marker replace an IdX-negative anti-HEL population appearing early after immunization. This replacement, along with the results in this report, indicates that idiotype selection *per se* plays a pivotal role in the predominance of the IdX-HEL marker during the secondary anti-HEL response. This selection is presumably mediated by an idiotype-specific T cell (IdXTh); in recent work it has been demonstrated that such IdXTh cells are necessary to complement antigen-specific T cells (AgTh) for the optimal induction of an *in vitro* anti-HEL response¹³. The activation of AgTh cells of a particular specificity under the influence of the major histocompatibility complex, in concert with antigen-bridging constraints in T-B collaboration, can explain the prevalent epitypic specificity of the B cell response. This combination of potent idiotype selection by IdXTh cells and specificity selection by AgTh cells would rationalize the common finding of convergence between idiotype and antigen specificity. Occasional utilization of less common T-helper cell clones could allow idiotype to be associated with antibody of different specificity, as we have noted here and as observed in the seminal findings of Oudin and his colleagues^{1,2}.

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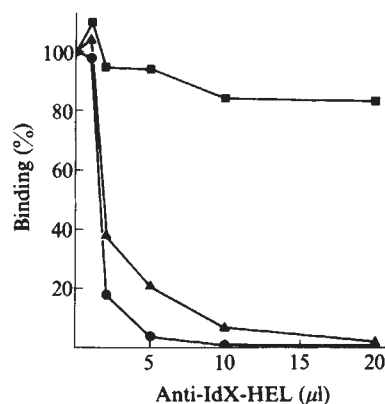


Fig. 1 Inhibition of hybridoma anti-HEL binding to ¹²⁵I-HEL by anti-idiotype. Anti-IdX-HEL was raised in guinea pigs using affinity-purified monoclonal anti-HEL antibody that was obtained by fusing immune B10.A lymph node cells with the BALB/c myeloma, P3x63Ag8. The resulting antiserum was repeatedly passed over normal (B10.AxBALB/c)F₁ Ig columns. Inhibition curves with anti-IdX-HEL were obtained by adding increasing amounts of anti-idiotype to tubes containing a dilution of hybridoma anti-HEL capable of binding 40–50% of 10ng ¹²⁵I-HEL. Following incubation, the Ig fraction in each tube was precipitated with 18% (w/v) Na₂SO₄, the precipitates were washed extensively, and the amount of bound ¹²⁵I-HEL was determined using a Nuclear Chicago γ-counter. Data are presented as the per cent of binding obtained in the absence of anti-idiotype. All values have been corrected for background binding in tubes containing anti-IdX-HEL and ¹²⁵I-HEL alone. ■ hybridoma antibody 2G3; ▲ hybridoma antibody 6D7; ● hybridoma antibody 5E4.

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Effect of cyclosporin A on T-dependent and T-independent immunoglobulin synthesis *in vitro*

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An immunosuppressive cyclic polypeptide, cyclosporin A (CyA), isolated originally from two species of fungi¹, prolongs organ allograft survival in several species^{2–7}. However, virtually nothing is known about the pharmacokinetics of this substance and its mode of action in man. We have quantified the impact of CyA on T-dependent and T-independent immunoglobulin (Ig) synthesis of human blood leukocytes *in vitro*, and on the proliferating capacity of the interacting lymphoid cell populations—T cells, B cells, T_H and T_H cells. CyA inhibited all responses at approximately equal concentrations. The blast cells rather than resting lymphocytes and/or Ig-synthesizing plasma cells were incapacitated by the drug. As the phagocytosis of accessory macrophages was inhibited only at a 100–1,000-fold higher drug concentration, we conclude that CyA has a relatively specific direct effect on human T and B blast cells *in vitro*.

Pokeweed mitogen (PWM) induces a T-dependent Ig-synthesis in human lymphocytes *in vitro*⁸, whereas *Staphylococcus aureus* bacteria act in similar conditions as T-independent mitogens⁹. Present fractionation methods enable adequate separation of the participating lymphoid cell populations and the evaluation of the effect of the drug on the different populations separately.

Human mononuclear leukocytes were isolated via Ficoll-Isopaque (FIP, Pharmacia) density centrifugation¹⁰, treated with iron powder plus magnet¹¹, washed and plated for culture. Microculture conditions in Falcon Microtest II tissue culture plates with 3×10^5 responder cells and 200- μ l volume were used. Pokeweed mitogen (50 μ g ml⁻¹, Gibco), or heat-killed, formalin-fixed *S. aureus* Cowan I bacteria⁹ at a concentration of 100×10^6 organisms ml⁻¹, were used as mitogens. CyA, which is insoluble in water, was first dissolved in absolute ethanol and added to cultures at indicated final concentrations. All cultures containing CyA contained an equal amount (0.13%) of ethanol, and for controls corresponding stimulations in the absence and in the presence of ethanol were used. The proliferative activity in cultures was measured by 6-h tritiated thymidine (³H-TdR) uptake (2 μ Ci ml⁻¹, specific activity 6.7 Ci mmol⁻¹; NEN) on the fourth culture day, when the peak proliferative response took place. To quantify the viable cell number, the blastogenic response and the rate of intracellular Ig synthesis, 10^4 chicken red blood cells (ChRBC) were added per culture well before terminating the cultures. The cell viability and the blastogenic response were assessed from May-Grünwald-Giemsa-stained cytocentrifuged cell smears¹¹. The degree of intracellular Ig synthesis was analysed from similar smears with fluorescein isothiocyanate-conjugated, 1:30 diluted, polyvalent anti-

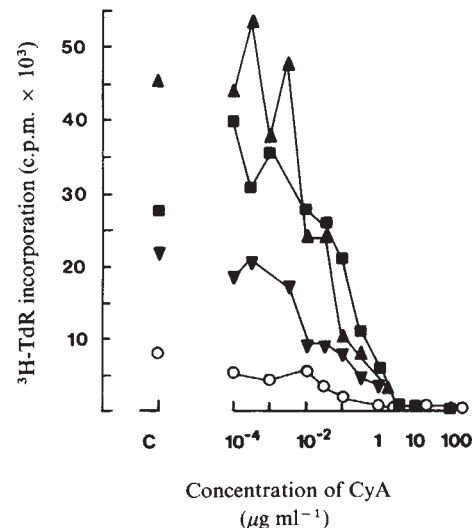


Fig. 1 Effect of increasing concentrations of CyA on the proliferative response of T- (■), T_μ- (▼), T_γ- (▲), and B-enriched (○) blood white cell populations to PWM. C indicates PWM-stimulated control cultures in the presence of 0.13% of ethanol. Mean incorporation in controls containing no mitogen was approximately $1-3 \times 10^2$ c.p.m.

human Ig (anti-IgM, -IgA, and -IgG; Dakopatts) using standard fluorescence technology. The results were expressed as number of blast cells, lymphocytes or cells containing intracellular Ig per 100 (autofluorescent) ChRBC. The release of Ig was quantified by indirect protein A (Pharmacia) plaque assay on the sixth culture day¹², and expressed as plaque-forming cells (PFC) per 10^5 cultured cells.

Fractionation of T and B cells and the T_μ and T_γ subsets of T cells was modified from Moretta *et al.*¹³: FIP-isolated iron powder-treated blood mononuclear cells were rosetted with 2-aminoethyl-isothiouonium bromide-hydrobromide-treated sheep red cells¹⁴ and centrifuged over FIP. The interphase (B-enriched) fraction contained >95% lymphocytes and 1–2% of monocytes. Approximately 80% of the lymphocytes expressed surface Ig. The red cells from the bottom (T-enriched) fraction were lysed¹¹ and the white cells were rosetted with IgG-coated erythrocytes as described¹³. After another centrifugation over FIP, the T_μ-enriched fraction was collected from the interphase while the bottom fraction was enriched for T_γ. In three separate experiments we found that the B cells, without recombination with T cells, were entirely unable to synthesize Ig after stimulation with PWM. When the B cells were recombined with T cells, the response was reconstituted. Recombination with T_μ cells induced an even higher Ig response, whereas recombination with the T_γ-enriched population was distinctly inferior in helper capacity. This indicates enrichment of helper cells in the former and either lack of helper cells and/or enrichment of suppressor cells in the latter T-cell fraction.

Mononuclear phagocytes were enriched via fractionation over a discontinuous Percoll gradient. Percoll (Pharmacia) was mixed with 10% fetal calf serum (FCS) containing RPMI-1640 medium (Gibco) at the following concentrations (%): 37.5, 40.0, 42.5, 45.0 and 47.5, and layered in five layers. 100×10^6 FIP-isolated blood leukocytes were layered in the RPMI-FCS medium on the top layer, the tubes were centrifuged for 45 min at 267g and the fractions from the five interphases were recovered. Mononuclear phagocytes were distributed primarily at the first interphase, where 90–95% of all leukocytes were monocytes according to morphological criteria.

In four experiments we stimulated the blood leukocytes with PWM or *S. aureus* bacteria, and quantified the rate of ³H-TdR incorporation, intracellular Ig synthesis and release of Ig into culture medium (Table 1). CyA suppressed all responses at

Table 1 Effect of CyA on PWM- and staphylococcus-induced *in vitro* responses of unfractionated blood cells

Test parameter and (day of assay)	Mitogen	Expt	No eth*	Eth†	Concentration of CyA ($\mu\text{g ml}^{-1}$)					
					10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10
$^3\text{H-TdR}$ incorporation (c.p.m. $\times 10^3$) (Day 4)	PWM	1	12.4	14.9	18.0	14.0	12.3	5.7	3.0	0.2
		2	23.2	26.7	23.0	21.9	22.2	16.5	13.2	0.2
	<i>Staphylococcus</i>	1	9.8	10.6	10.5	10.4	8.1	2.9	1.7	0.2
		2	13.9	13.8	13.1	14.6	13.0	9.1	8.0	0.1
	—	1b	0.8	1.3	1.1	0.9	0.8	0.4	0.4	0.1
		2	2.7	2.8	1.5	0.9	0.9	0.7	0.4	0.1
Intracellular Ig-containing cells (per 100 ChRBC) (Day 6)	PWM	3	44	52	62	49	37	17	1	0
	<i>Staphylococcus</i>	3	37	59	42	32	30	7	3	0
	—	3	3	2	1	1	0	4	0	0
Ig-releasing cells (PFC per 100,000 cultured cells) (Day 6)	PWM	1	258	270	285	276	138	60	30	6
	<i>Staphylococcus</i>	1	201	202	216	180	132	54	12	0
	—	1	10	16	12	10	10	4	6	0

* No ethanol present in the control culture.

† 0.13% of ethanol was present in this control culture and in all CyA-containing cultures.

Table 2 Effect of CyA on the viability of lymphocytes and blast cells in PWM- and *S. aureus*-stimulated culture of unfractionated blood cells

Mitogen*	Cell per culture (per 100 ChRBC)	No eth†	Eth‡	Concentration of CyA ($\mu\text{g ml}^{-1}$)					
				10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10
PWM	Blasts	333	355	225	327	275	120	109	5
	Lymphocytes	886	676	999	786	861	1,010	868	378
<i>Staphylococcus</i>	Blasts	147	176	166	108	65	56	29	0
	Lymphocytes	1,394	996	937	965	755	1,032	1,111	272

* Counts performed on the 6th day of culture.

† Control without ethanol.

‡ 0.13% of ethanol was present in this control culture and in all CyA-containing cultures.

Table 3 Effect of CyA on the phagocytic activity of cultured blood monocytes

Expt	No eth*	Eth†	Concentration of CyA ($\mu\text{g ml}^{-1}$)‡					
			10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10
1	6.5 ± 0.8	7.0 ± 0.9	6.3 ± 0.8	5.5 ± 0.5	6.7 ± 0.7	4.8 ± 0.6	4.5 ± 0.6	2.8 ± 0.7
2	6.0 ± 0.5	5.0 ± 0.6	5.5 ± 0.5	4.9 ± 0.5	5.0 ± 0.6	4.0 ± 0.4	4.9 ± 0.4	2.0 ± 0.4

* No ethanol present in control culture.

† 0.13% ethanol added to control culture.

‡ Number of ingested staphylococci per cell after culture of the blood monocytes for 48 h in the presence of various concentration of CyA. Mean numbers of triplicate determinations $\pm$ s.d. Exposure time of staphylococci, 30 min; CyA present in the culture medium also during phagocytosis.

equal concentrations, regardless of whether PWM or staphylococci were used as mitogen.

Blood leukocytes were then enriched for T and B cells, and the T cells further to T_μ and T_γ cells. The effect of the drug was assessed separately on the proliferative response of the different cell populations. In all three experiments the drug suppressed at equal concentrations the proliferative response of T, T_μ and T_γ and B-enriched cell populations to PWM (Fig. 1). Whereas a distinct suppression of the blastogenic response (Table 2) was observed already at a concentration of 10^{-3} – 10^{-2} $\mu\text{g ml}^{-1}$, the viability of resting lymphocytes was not decreased until a concentration of 1–10 $\mu\text{g ml}^{-1}$ was reached.

Because both mitogens are monocyte-dependent, a possibility still existed that CyA affects through the accessory cells, that is, through the monocytes present in the culture. Blood monocyte-enriched population was therefore cultured for 48 h in the presence of different concentrations of CyA, and 200×10^6 staphylococci were added per culture. After an additional 30-min incubation, coverslips were removed from the bottom of the culture vessels, washed, stained and the average number of intracellular staphylococci was quantified (Table 3). Prior culture with CyA has virtually no effect on the phagocytic

activity of blood monocytes up to a concentration of 1 $\mu\text{g ml}^{-1}$. At 10 $\mu\text{g ml}^{-1}$, the rate of phagocytosis was reduced by $\sim 50\%$.

Altogether, CyA suppressed at equal concentrations both T-dependent and T-independent Ig production. The distinctly inhibitory concentration *in vitro*, 1×10^{-1} $\mu\text{g ml}^{-1}$, was somewhat smaller than the expected maintenance concentration in the blood after peroral administration at the recommended dose of 17 mg per kg per day (ref. 7). Moreover, all participating populations of lymphoid cells—T helper cells, T suppressor cells and B cells—were equally sensitive to this drug, and the drug suppressed far better the blastogenic response than killed resting lymphocytes in cultures. Because blood monocyte phagocytosis was not affected by previous culture with CyA, we consider it unlikely that the drug effect is mediated through the accessory mononuclear phagocytes^{15,16}.

Although species differences may exist, our results contradict the finding of White *et al.*¹⁷ on porcine lymphocytes that T cells are more sensitive than B cells to CyA *in vitro* and those of Gordon *et al.*¹⁸ who demonstrated that colony formation by transformed human 'B lymphocytes' is marginally less sensitive to CyA than colony formation by (a fraction of) T lymphocytes. However, our results are compatible with those of Borel *et al.*²

who demonstrated that haemagglutinating antibody response in the mouse was suppressed at approximately equal doses compared to skin graft rejection, and with the observation of Allison *et al.* (unpublished, cited in ref. 4) that T and B blastogenic responses of human lymphocytes are affected at equal drug concentrations. The rather selective effect of CyA on T and B blast cells is compatible with the 'decloning' hypothesis suggested for CyA as a possible mode of action in certain species^{4,19}, though not yet in man.

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An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it

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The immune system does not normally react against self components. Originally, it was postulated that self-reactive cells were somehow deleted or blocked¹. More recent thinking^{2,3} is that such cells are suppressed by regulatory networks similar to those limiting the immune response against non-self determinants. Both mechanisms may exist^{4,5}. I describe here a type of suppression more closely related to the first postulate. In the *in vitro*, one-way, mixed lymphocyte reaction (MLR), cytotoxic T-lymphocyte precursor cells (CLP) from the responder population give rise to cytotoxic T-lymphocytes (CL) capable of lysing target cells from the stimulator population⁶⁻⁸. A sub-population of cells in the spleen of athymic nude mice can, when added to such cultures, inactivate CLP capable of recognizing either the H-2 antigens or TNP modifications of the nude spleen. Regarding the nude spleen cells, activation of self-reactive cells is being prevented.

Table 1 presents measurements of the cytotoxic activity produced in various MLRs which demonstrate the suppression phenomenon. In experiment 1, line 1, BALB/c (H-2^d) lymph node (LN) cells produce a good cytotoxic response against both irradiated RNC (H-2^k) spleen cells and irradiated B10 (H-2^b) spleen cells. Addition of BALB/c.nu spleen cells to the cultures has little effect on either response (line 2). However, addition of RNC.nu spleen cells leads to a significantly lower cytotoxic response against the RNC spleen cells but not against the B10

spleen cells, that is, suppression is observed when the added nude spleen cells are syngeneic to the stimulator cells (line 3). The ability of the added nude spleen cells to cause suppression is destroyed by subjecting them to one freeze-thaw cycle (line 4), implying that the suppression results from a physiological process. These results confirm previous, more extensive studies⁹ in which it was shown that B.nu or (A×B)F₁.nu spleen cells suppress an A anti-B response but not an A anti-C response where A, B and C represent three H-2-different mouse strains.

An anti-TNP-modified self response can also be suppressed. Addition of RNC.nu spleen cells to RNC anti-RNC-TNP cultures suppressed the response if the RNC.nu spleen cells were also TNP-modified (Table 1, expt 2). The same nude cells, whether TNP-modified or not, were equally competent at suppressing a BALB/c anti-RNC response. Similar results have been obtained using B6.nu spleen cells in the B6 anti-B6-TNP response and BALB/c.nu spleen cells in the BALB/c anti-BALB/c-TNP response. Controls established that the effector

Table 1 Suppression of cytotoxic responses by added nude spleen cells

Expt	Added nude spleen cells	Cytotoxic activity	
		BALB/c anti-RNC response	BALB/c anti-B10 response
Expt 1	0	196 ± 28	114 ± 25
	BALB/c	228 ± 65	158 ± 25
	RNC	86 ± 8*	111 ± 23
	RNC (freeze-thaw)†	168 ± 19	NT
Expt 2	0	192 ± 22	201 ± 26
	RNC	183 ± 18	38 ± 8*
	RNC-TNP	77 ± 16*	41 ± 7*
		BALB/c anti-BALB/c-TNP response	
Expt 3	0	55 ± 12	
	BALB/c-TNP mixture‡	15 ± 7*	
		60 ± 10	
Expt 4	0	(RNC×C57BL/6)F ₁ anti-RNC-TNP response	(RNC×C57BL/6)F ₁ anti-B6-TNP response
	RNC	216 ± 47	50 ± 10
	RNC-TNP	216 ± 27	54 ± 10
		109 ± 16*	27 ± 5*

Each entry is the mean ± s.e.m. cytotoxic activity for a group of eight microcultures, each culture containing LN cells as responder cells, 3×10^5 irradiated (1,500 rad ¹³⁷Cs γ rays) spleen cells as stimulator cells and 10^5 (expt 1 and expt 3) or 3×10^5 (expt 2 and expt 4) nude spleen cells being tested for their ability to suppress the response of the culture. For the anti-H-2 responses (BALB/c anti-RNC, BALB/c anti-B10), 3×10^4 responder LN cells were used; for the remaining cultures, 2×10^5 responder LN cells were used. When required, TNP-modified cells were produced by the method of Shearer *et al.*¹⁷ as described previously¹⁸. Each experiment included control groups containing nude spleen cells and stimulator cells but no responder LN cells. None of these control groups developed significant cytotoxic activity. Each culture was in 0.2 ml of α-minimal essential medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol and 10 mM HEPES buffer as described in detail elsewhere⁹. After 5 days of incubation at 37 °C, cultures were assayed for cytotoxic activity by addition of 3×10^3 ⁵¹Cr-labelled target cells obtained from a 2- or 3-day culture of spleen cells identical to the stimulator cells incubated with concanavalin A ($2 \mu\text{g ml}^{-1}$). Fractional specific ⁵¹Cr release, *p*, defined as (observed release - spontaneous release)/(total releasable - spontaneous release) was measured as previously described⁹ and re-expressed as cytotoxic activity = $-100 \ln(1 - p)$. This transformation re-expresses the ⁵¹Cr release so that the cytotoxic activity is directly proportional to the number of CL present rather than the number of target cells destroyed¹⁹. An entry of 10 corresponds to 10% specific ⁵¹Cr release; an entry of 100 to 63% specific ⁵¹Cr release. NT, Not tested.

* Response significantly less ($P < 0.05$) than for the corresponding group of cultures not containing added nude spleen cells.

† The RNC.nu spleen cells were frozen and thawed once before addition to the cultures.

‡ Cultures identical to those of the preceding two lines, that is, with and without added BALB/c.nu-TNP spleen cells, were mixed immediately before assaying for cytotoxic activity.

cells produced were T-cells that lysed syngeneic cells only after TNP modification.

The suppression produced by adding nude spleen cells could occur at one or more of several levels of the MLR and subsequent cytotoxicity assay: (1) a reduction in the number of CLP activated; (2) a reduction in the number of CL produced per activated CLP; or (3) a reduction in the measured cytotoxic activity per CL either as a result of suppression of the lytic efficiency per CL or as a result of competitive inhibition of the lysis of the labelled target cells by remaining unlabelled nude spleen cells. Experiment 3, Table 1, provides evidence against the last possibility: adding suppressed cultures to non-suppressed cultures just before the assay for cytotoxicity produced no decrease in the measured cytotoxic activity. To distinguish between possibilities (1) and (2), limiting dilution analysis was used to measure both the frequency of CLP (f_{CLP}) and the relative number of CL produced per activated CLP in the presence and absence of nude suppressor cells. In the culture conditions used, the CLP is the least frequent required cell so that failure of a culture to respond indicates that the culture contained no CLP^{8,10}. The probability, P , of this happening is, from the first term of the Poisson distribution, given by $P = \exp(-f_{CLP}N)$ where N is the number of LN cells cultured. The cytotoxic activity developed in replicate RNC anti-RNC-TNP cultures containing varying numbers of RNC LN responder cells, fixed numbers of RNC.nu spleen cells with or without TNP modification, and fixed numbers of RNC-TNP stimulator cells, was measured and each culture was scored as responding or non-responding. Figure 1a shows the fraction of non-responding cultures and Fig. 1b the mean cytotoxic activity per culture, both as a function of N . The values for f_{CLP} determined from Fig. 1a are shown in Table 2 (line 1). The value for f_{CLP} is about two and a half times lower when the added nude spleen cells have been TNP-modified. From the slopes of the lines in Fig. 1b and from the measured CLP frequency, one can calculate the mean cytotoxic activity produced by the clone of CL from a single CLP (Table 2). It is significantly greater in the presence of suppression. Possibly the suppressor cells can suppress regulator cells, limiting the magnitude of the response² as well as preventing CLP activation. Note that this effect tends to reduce the overall observed suppression in cytotoxic activity. Equivalent results were obtained on analysing the BALB/c anti-BALB/c-TNP response (expt 2, Table 2).

The suppression observed in an anti-H-2 response also results from a reduction in the number of CLP activated (expt 3, Table 2). RNC.nu spleen cells were used to suppress a BALB/c anti-RNC response. To obtain a population of nude cells which would not suppress, but at the same time provide the accessory cells required to meet the conditions of the limiting dilution assay⁸, use was made of a previous observation⁹ that slowly-sedimenting nude spleen cells contain the required accessory cells but are relatively free of suppressive activity, whereas rapidly-sedimenting cells are enriched in suppressive activity. Thus, the CLP frequency measured in the presence of nude spleen cells (line 1) is about half that measured in the presence of slowly-sedimenting (2–4.5 mm h⁻¹) nude spleen cells (line 2). Addition of rapidly-sedimenting (4.5–9 mm h⁻¹) nude cells to the slowly-sedimenting nude cells restores the suppression.

The cytotoxic response against syngeneic target cells which have been virus- or TNP-modified is known to be H-2 restricted, that is, the CL obtained produce preferential lysis of target cells sharing both the modification (virus or TNP) and H-2 with the original stimulator cells^{11,12}. I therefore investigated whether TNP-modified nude spleen cells could suppress an anti-TNP response on an H-2-different background. Care was taken to avoid possible allogeneic effects by working only with parent-F₁ strain combinations. In expt 4, Table 1, RNC.nu spleen cells, with and without TNP modification, were added to either an (RNC×C57BL/6)F₁ anti-RNC-TNP response or an (RNC×C57BL/6)F₁ anti-C57BL/6-TNP response. The RNC-TNP-modified nude cells suppressed both responses about twofold. Similar results were obtained on adding RNC.nu

Table 2 Frequency of CLPs activated in presence and absence of suppressor cells

Expt	Response	Added nude spleen cells	Precursor frequency per 10 ⁵ LN cells	Cytotoxic activity per clone
1	RNC anti-RNC-TNP	RNC RNC-TNP	182 (135–246) 73 (53–100)	5.3 ± 0.5 6.7 ± 0.7
2	BALB/c anti-BALB/c-TNP	BALB/c BALB/c-TNP	105 (65–168) 37 (24–58)	7.1 ± 0.7 8.7 ± 0.9
3	BALB/c anti-RNC	RNC (unfractionated) RNC (2–4.5 mm h ⁻¹) RNC (2–4.5 mm h ⁻¹) +RNC (4.5–9 mm h ⁻¹)	594 (413–853) 1,140 (877–1482) 468 (348–629)	11.5 ± 1.2 5.4 ± 0.5 8.3 ± 0.8

The frequency of CLP and cytotoxic activity per clone were measured by limiting dilution analysis as in Fig. 1; experiment 1 is the result from analysing this figure. The frequency was calculated using the method of Porter and Berry²⁰, the bracketed numbers after the entry giving 95% confidence limits. Experiment 2 was performed in an identical manner. In experiment 3, the added RNC.nu spleen cells were fractionated by velocity sedimentation²¹ and either 3 × 10⁵ unfractionated, or 3 × 10⁵ (2–4.5 mm h⁻¹), or 3 × 10⁵ (2–4.5 mm h⁻¹) plus 10⁵ (4.5–9 mm h⁻¹) nude spleen cells tested for their ability to produce suppression.

spleen cells, with and without TNP modification, to an (RNC×C57BL/6)F₁ anti-(RNC×C57BL/6)F₁-TNP response and measuring the response against RNC-TNP and C57BL/6-TNP target cells. I conclude that the nude spleen suppressor cells show at most a slight preference for suppression of a TNP-modified self response in which the H-2 of the nude cells and stimulator cells is shared. In experiments using the same micro-culture technique as used here to analyse TNP cross-reactivity at the level of single clones of CL (ref. 18), it was found that at least 40% of clones specific for the TNP-modified stimulator could also lyse a particular H-2-unrelated, TNP-modified target cell. Thus, the apparent lack of H-2 restriction seen here might merely reflect cross-reactivity at the level of the CLP, the presumed site of action of the suppressor.

What is the mechanism of the observed suppression? To obtain suppression, the added nude cells must carry the antigen against which the response is directed, H-2 in the case of an anti-H-2 response and TNP in the case of an anti-TNP response (Table 1). The reduction in cytotoxic activity results from a reduction in the number of CLP activated rather than the number of progeny CL produced per activated CLP (Table 2). Only a subpopulation of all the cells in nude spleen is capable of inducing the suppression (Table 2, expt 3 and ref. 9). The simplest explanation for these observations is that nude spleen contains a cell subpopulation which, when recognized by a CLP, sends a signal to the CLP which inactivates it. Whether the inactivation is reversible or permanent, that is, whether, using the terminology of enzymology, inhibition is competitive or non-competitive, is not firmly established at the present time although attempts to rescue inactivated CLP have so far been unsuccessful. Not all CLP can be inhibited. Although the cytotoxic activity developed in a primary MLR can be strongly inhibited, the inhibition is never complete and a secondary MLR cannot be inhibited at all (J. J. Rusthoven and R. G. M., unpublished observation). The inactivating signal could well be a factor although, if so, it must be short range or good fits would not have been obtained in the Poisson limiting dilution analysis (Fig. 1). In addition, attempts to isolate such a factor have so far been unsuccessful. The most novel feature of the model just outlined is the direction of recognition triggering the suppression: the CLP triggers its own suppression by recognizing the suppressor cell. An alternative point of view is that the suppressor cells have a repertoire of receptors capable of recognizing receptors which can recognize self antigens, that is, the suppression takes place through recognition of anti-self idiotypes¹³. Against this model is the observation (Table 1) that the suppressor cell can suppress an anti-self-TNP response only after the suppressor cell has been itself TNP-modified. It is

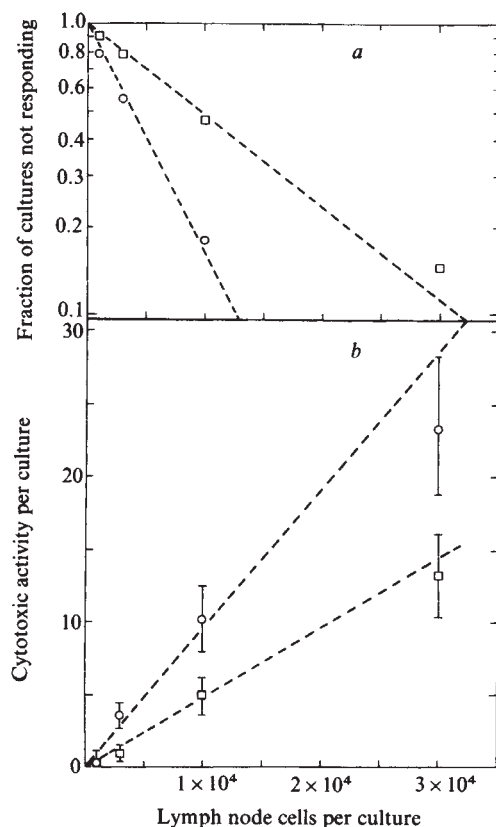


Fig. 1 Limiting dilution analysis of the RNC anti-RNC-TNP response in the presence of either RNC.nu (○) or RNC.nu-TNP (□) spleen cells. Groups of from 32 to 48 microcultures were set up and assayed as in Table 1. Each group contained RNC LN responder cells as indicated on the abscissa, 3×10^5 irradiated (1,500 rad) and TNP-modified RNC spleen stimulator cells, and 3×10^5 RNC.nu spleen cells either with or without TNP modification. The fraction of non-responding cultures as a function of the number of LN cells is plotted in *a*. A culture was scored as responding if the measured ^{51}Cr release was greater than the mean ± 2 s.e.m. measured for a group of cultures from which LN responder cells were omitted. The mean cytotoxic activity per culture (including both responding and non-responding cultures) is plotted in *b* as a function of the number of LN responder cells cultured.

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Intercellular communication patterns are involved in cell determination in early molluscan development

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The formation of specialized intercellular junctions, allowing the passage of low-molecular weight regulatory molecules, has been considered as a possible mechanism for regulating embryonic development^{1,2}. No direct evidence for this concept has been found in early development. In the mollusc *Patella vulgata* it was demonstrated that cell positioning and specific cellular interactions are key factors in the control of early development^{3,4}. We have now investigated the pattern of intercellular communication during early development of this embryo by intracellular iontophoresis of the fluorescent dye Lucifer Yellow CH. We demonstrate that the formation of regional- and temporal-specific cell-to-cell coupling is correlated with the determination of the mesentoblast—the stem cell of the mesoderm—and the establishment of dorso-ventral polarity.

In the *Patella* embryo, bilateral symmetry is indicated from the 4-cell stage by the appearance of a distinct cross-furrow between the blastomeres B and D and a minimal one between the blastomeres A and C (Fig. 1)^{3,4}. At the early 32-cell stage the macromeres of each quadrant (3A–3D) still have the capacity to develop into mesentoblast or to form entodermal structures. At this stage these macromeres protrude into the embryo after the withdrawal of the originally centrally placed second quartet cells. As a consequence of their relative positions only one of the cross-furrow macromeres (then denoted as 3D) is in contact temporarily with the overlying animal micromere quartet (1a¹¹–1d¹¹), and it is this event which determines that this macromere will form the mesentoblast and establishes dorso-ventral polarity perpendicular to the cross-furrow. Cell deletion experiments have demonstrated that such contact between the

difficult to see how TNP modification could create a new receptor.

The unique properties of the suppressive activity described here are that the suppression is specific for antigens carried by the suppressive cell population and that the suppression is at the level of CLP activation in the responding cell population. Other tissues besides nude spleen have been screened for suppressive activity with the above properties. It is not seen in normal spleen^{9,14} but is found in both normal bone marrow¹⁴ and in thymus¹⁵. The suppressive activity found in bone marrow may represent a precursor population which seeds to thymus in a normal mouse but seeds to spleen in the athymic nude mouse. In the thymus, the suppressor cell could inactivate cells recognizing it before such cells are exported to the periphery. Such a mechanism is required in models for the somatic development of the specificity repertoire in which reactions against self antigens, particularly self H-2¹⁶ are instrumental in driving the somatic variation of the repertoire. Cells reactive against foreign antigens might also be inactivated if the foreign antigens were incorporated on the surface of the suppressor cell.

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macromere 3D and the micromeres $1a^{11}-1d^{11}$ is a prerequisite for these determinative events⁴.

The intercellular transfer of low-molecular weight fluorescent dyes and intercellular electrical coupling are both considered as indicators for the presence of functional cell-to-cell pathways, structurally recognizable as gap junctions in the apposed junctional membranes^{5,6}. Complex intercellular communication patterns can be easily recognized from intercellular transfer of fluorescent dyes. Lucifer Yellow CH (molecular weight 457.3) is extremely suitable for this purpose⁷, and its intercellular transfer was found to be associated with the presence of gap junctions and electrical coupling^{8,9}.

As we were interested in the possible role of intercellular coupling in mesentoblast determination, we restricted the dye application to the vegetal macromeres, both cross-furrow and non-cross-furrow, from the 4-cell stage to the 32-cell stage. Because the dorso-ventral polarity of the embryo is absent at early cleavage stages, identification of the injected cell had to be made by the unequal cleavage pattern at the sixth cleavage³. In all cases Lucifer Yellow CH spread through the entire cell within 5 min. No effects were observed on subsequent cell division.

Until the 16-cell stage initial dye spread was restricted consistently to the impaled cell, demonstrating the absence of functional intercellular communication pathways (Fig. 1a-d). This situation changed completely after the fifth cleavage. At the 32-cell stage, as the vegetal macromeres start to protrude into the embryo, intercellular junctions that allow the passage of Lucifer Yellow CH form between the vegetal macromeres and their neighbouring cells (Fig. 2a, b), with the exception of the junctional membrane between the macromere 3D and the blastomere $2d^2$, its neighbour on the future dorsal side (Figs 2c, d and 3). This communication pattern demonstrates the establishment of dorso-ventral polarity at the 32-cell stage. No dye transfer was observed from the macromeres 3A, 3B or 3C to animal cells. In contrast, the macromere 3D becomes exclusively coupled to the central animal micromere quartet $1a^{11}-1d^{11}$ at this stage (Fig. 2e, f). Although the non-central micromeres $1a^{12}-1d^{12}$, surrounding the central micromeres $1a^{11}-1d^{11}$, will come temporarily into contact with any of the macromeres at the early 32-cell stage, no dye transfer was observed between them. We conclude that only the central micromere quartet is able to become coupled to a macromere. By observing whole embryos we could not exclude the possibility that there is minimal dye transfer from the macromeres to their second neighbouring cells. However, histological sections of embryos fixed after dye injection confirmed that dye transfer from the macromeres is restricted to their neighbouring cells (Fig. 3).

Our results further demonstrate the binding of Lucifer Yellow CH to cytoplasmic components, as suggested previously⁷. With dye application at the 4-cell stage, fluorescence is only detectable in all daughter cells at later stages (Fig. 1e-h), in contrast to the dye spread observed immediately on dye injection into a macromere at the 32-cell stage (Fig. 2). From such considerations we estimated that cytoplasmic binding prevents further transfer of Lucifer Yellow CH in these embryos within about 30 min.

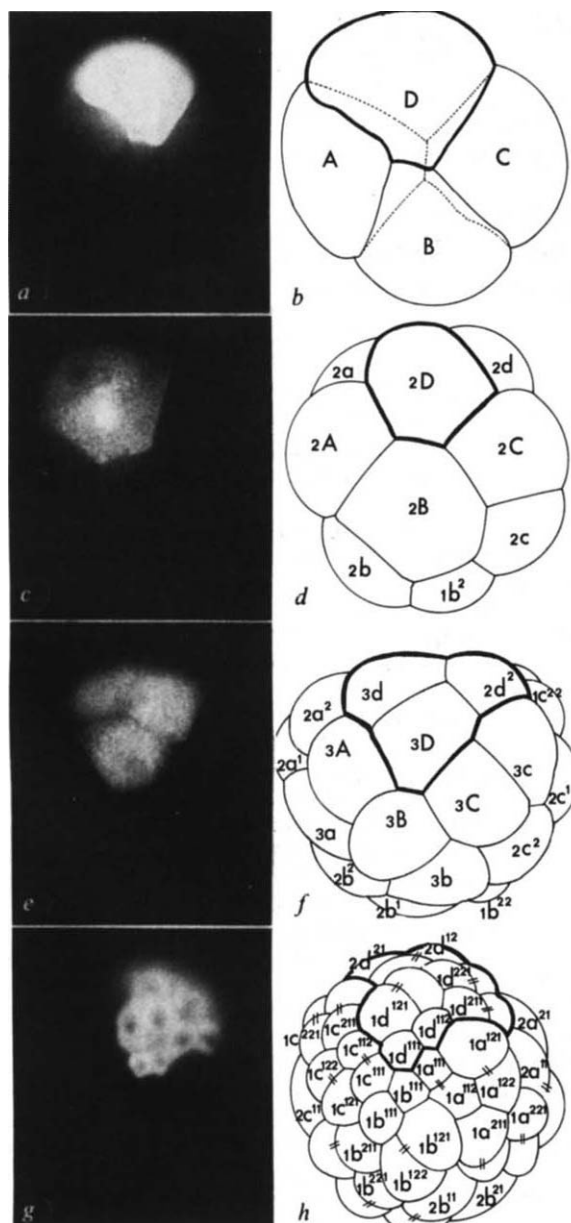


Fig. 1 Left column: micrographs of unfixed embryos of *Patella vulgata* after iontophoresis of Lucifer Yellow CH into one of the two cross-furrow cells at the 4- and 16-cell stage $\times 200$. Right column: diagrams of the corresponding cell patterns. Before the mid 32-cell stage the two cross-furrow quadrants cannot be distinguished. For convenience the labelled quadrant has then been designated as D. a, b: 4-cell embryo 20 min after labelling of the D-cell; no dye transfer to adjacent cells. c, d: 16-cell embryo 20 min after labelling the macromere 2D; no dye transfer to adjacent cells. e, f: Vegetal view of the same embryo as in a at the onset of the 32-cell stage; only the progeny of D ($3D$, $3d$, $2d^2$; others not visible) have received the dye. g, h: Animal view of the same embryo as in a at the 60-cell stage; only the micromeres of the injected quadrant have received the dye at successive cell divisions. *P. vulgata* used in this study were collected in the region of Point aux Oies, near Wimereux in France. Eggs were obtained, fertilized and cultured as described elsewhere³. The eggs were decapsulated by a brief treatment with acidified sea water, pH 3.8. Conventional glass microelectrodes were used for intracellular application of Lucifer Yellow CH. The tip of the micropipette was filled with a 3% aqueous solution of the dye whereas the remainder of the electrode system was filled with 3 M LiCl. Electrode resistances reached values greater than 200 M Ω . The dye was introduced iontophoretically into the cell by applying repetitive hyperpolarizing current pulses of 5 nA and 1.5 s with a time interval of 0.5 s for 3 min. The microelectrodes were connected to a high-impedance pre-amplifier equipped with a bridge circuit so that the membrane potential (-35 to -65 mV, depending on developmental stage) could be recorded as an indicator for proper cell penetration. During dye iontophoresis the embryos were observed in a dissecting microscope. The spread of fluorescence was observed in a Leitz Orthoplan epiluminescence fluorescence microscope with a $\times 32$ objective (excitation: 450–490 nm; emission: >515 nm). Photomicrographs were taken on Kodak High Speed Ektachrome or Kodak High Speed Recording Film, with exposure times of 15–30 s.

The present results are the first demonstration that the formation of specific intercellular communication patterns is involved in cell determination in early development. In *P. vulgata* the interaction between the central micromere quartet and a macromere at the 32-cell stage results in the determination of this macromere as the stem cell of the mesoderm and of the dorso-ventral polarity in the embryo. The reported formation of intercellular communication pathways, probably via gap junctions, is exactly correlated in time and space with these determinative events. In this embryo the relative positions of the macromeres, together with the capacity of the cells along

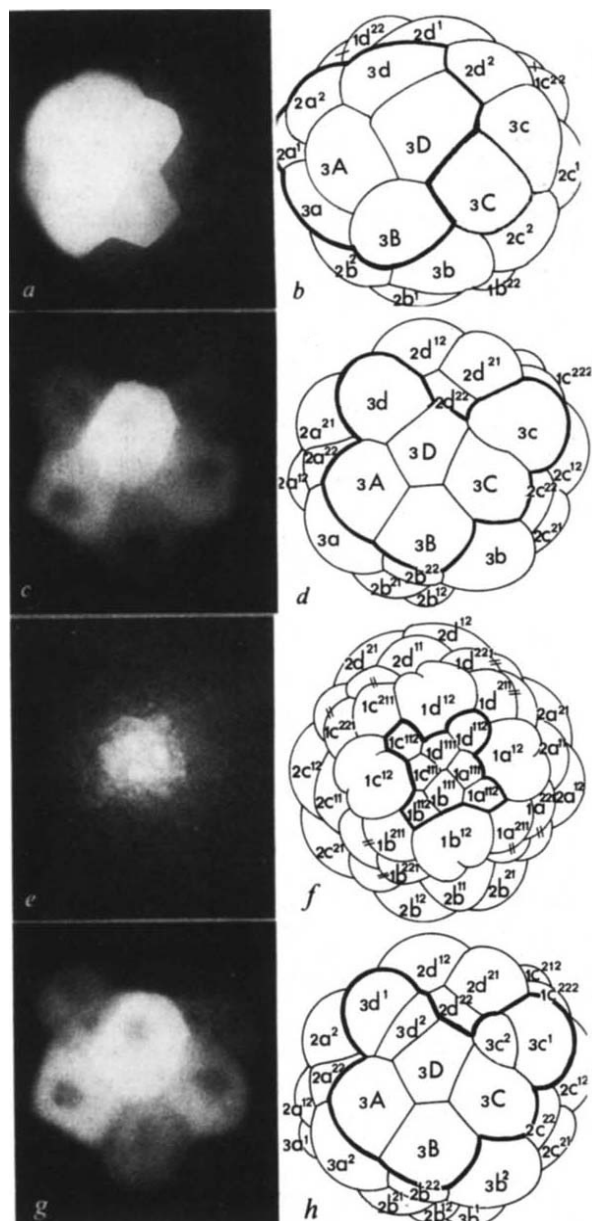


Fig. 2 Left column: micrographs of unfixed embryos of *P. vulgata* after iontophoresis of Lucifer Yellow CH into one of the four macromeres 3A–3D at the 32-cell stage $\times 200$. Right column: diagrams of the corresponding cell patterns. *a, b*: Vegetal view of a 32-cell embryo after labelling macromere 3A; dye transfer is restricted to all adjacent cells. *c, d*: Vegetal view of a 32-cell embryo after labelling the macromere 3D; dye transfer is restricted to adjacent cells, except $2d^2$. *e, f*: Animal view of the same embryo as in *c*, about 45 min later; dye transfer from the macromere 3D to the central animal micromeres. *g, h*: Vegetal view of the same embryo as in *c*, about 60 min later; no further transfer in comparison with *c*.

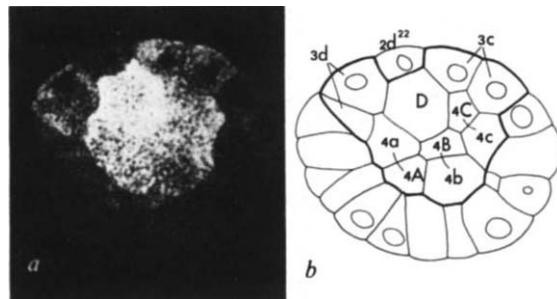


Fig. 3 *a*, Micrograph of an oblique section through an older stage of the same embryo as in Fig. 2c, e, g after fixation in 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.4. Dye is only detectable in cells adjacent to the injected macromere 3D (3A, 3B, 3C, 3d, 3c) and their progeny, except its dorsal neighbour $2d^{22}$. *b*, Diagram of the corresponding cell pattern $\times 200$.

the animal–vegetal axis to become coupled at the 32-cell stage, determine bilateral symmetry.

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Pharmacological specificity of brain histamine H_2 -receptors differs in intact cells and cell-free preparations

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Despite increasing evidence that histamine may be a neurotransmitter in brain, the functions of putative histaminergic neurones are just beginning to be known^{1,2}. Both H_1 - and H_2 -receptors³ seem to be present in brain^{4–7} and histaminergic neurones might control arousal mechanisms or carbohydrate metabolism, via H_1 -receptors^{8–11}. The actions mediated by cerebral H_2 -receptors are less clear mainly because available agonists and antagonists^{3,12–15} do not cross the 'blood–brain barrier'. However, on a histamine-sensitive adenylate cyclase from guinea pig brain homogenates with pharmacological properties similar to those of H_2 -receptors^{16,17} compounds like tricyclic antidepressants or promethazine were extremely potent antagonists^{18,19} suggesting that this might represent the molecular basis of clinical antidepressant activity¹⁹. Although electrophysiological²⁰ and behavioural²¹ studies partially confirmed that one of these compounds, amitryptiline, could block cerebral histamine receptors in the living animal, their potency as H_2 -antihistamines has never been precisely established in intact brain cell preparations. Histamine strongly stimulates cyclic AMP synthesis in brain slices but, in contrast with homogenates, both H_1 - and H_2 -receptors are involved^{4,5,22}. As compounds like promethazine are potent H_1 -antihistamines^{7,8}, we have developed a test specific for H_2 -receptors consisting of the stimulation of cyclic AMP accumulation by impromidine, a potent and highly selective H_2 -receptor agonist¹⁴, in slices from guinea pig hippocampus and using it find that H_2 -receptors differ considerably in their drug discriminatory properties from those mediating the stimulation of adenylate cyclase activity in homogenates.

The involvement of both H_1 - and H_2 -receptors in the cyclic AMP response to histamine in hippocampal slices was shown mainly by the biphasic rightward shift of concentration-response curves to this amine in the presence of low concentrations of mepyramine, a pure H_1 -antihistamine and the additive responses to H_1 - and H_2 -agonists²². This is further illustrated when considering the antagonism of histamine-induced accumulation of the nucleotide in slices of guinea pig hippocampus by promethazine, a potent H_1 -antihistamine²³, also recently shown to inhibit the brain histamine-sensitive adenylyl cyclase^{19,24}. Thus the response to a fixed concentration of histamine (50 μ M) is inhibited in a clearly biphasic manner by promethazine in increasing concentrations (Fig. 1). Approximately 50% of the response is inhibited with an IC_{50} of 0.13 μ M whereas the remaining is abolished, after a plateau, for much higher concentrations ($IC_{50} = 32.9 \mu$ M). A similar pattern of inhibition is obtained with cyproheptadine leading to IC_{50} values of 0.03 μ M and 12 μ M (not shown). Among the two components of the histamine response distinguished by promethazine, the first is likely to be mediated by H_1 -receptors as proposed earlier²², whereas the second is mediated by H_2 -receptors as shown by the antagonism of highly selective agonists of these receptors. One of them, impromidine, is a particularly useful tool being 40–50 times as potent as histamine on a variety of biological responses mediated by H_2 -receptors

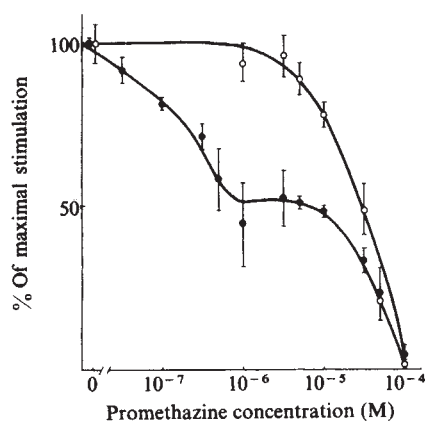


Fig. 1 Inhibition by promethazine of histamine- and impromidine-induced stimulation of cyclic AMP accumulation in slices from guinea pig hippocampus. Hartley guinea pigs were killed by decapitation and slices (250 μ m thick) were prepared from hippocampus. Pooled slices from four animals were incubated for 30 min at 37 °C in Krebs–Ringer bicarbonate medium (40 ml per g of tissue) under a constant stream of O_2 : CO_2 (95:5) in a Dubnoff metabolic shaker. Slices were then washed twice with fresh medium and 200 μ l aliquots (about 500 μ g protein) were distributed in incubation tubes containing promethazine. After 15 min at 37 °C under agitation, histamine or impromidine were added and a further 15-min incubation was performed. Incubations were terminated by sonication of the slices and heating for 10 min at 95 °C. Cyclic AMP concentration was determined by the protein kinase binding assay³⁹. Results are expressed as per cent of stimulation elicited in the absence of promethazine. Each point represents the mean $\pm$ s.e.m. of six values obtained in two separate experiments. The accumulation of cyclic AMP (in pmol per mg protein) was 64.0 ± 2.7 for 50 μ M histamine and 39.7 ± 2.2 for 1 μ M impromidine. Basal levels (11.6 ± 1.3 pmol per mg protein⁻¹) were not altered in the presence of promethazine in concentrations up to 100 μ M. The IC_{50} values for promethazine, established by log-probit analysis, were 0.13 μ M and 32.9 μ M against histamine, and 22.0 μ M against impromidine. Assuming competitive inhibition, the K_i values were calculated according to the equation⁴⁰ $K_i = IC_{50}/(1 + S/EC_{50})$, where S represents the concentration of histamine or impromidine. The EC_{50} value of histamine was 10 μ M (ref. 22) and that of impromidine, 0.16 μ M (mean of values obtained in seven experiments similar to that of Fig. 2). K_i values were 0.022 μ M against the first component of the response to histamine, 5.5 μ M against the second component and 3.0 μ M against impromidine. ●, Histamine (50 μ M); ○, impromidine (1 μ M).

Table 1 Comparison of the potency of several drugs on the histamine H_2 -receptor-mediated stimulation of cyclic AMP formation in slices and homogenates from guinea pig hippocampus

Drugs	Inhibition constants (K_i , μ M)	
	Slices	Homogenates
H_2-receptor antagonists		
Cimetidine	0.62	0.89* 0.60†
Metiamide	0.82	0.89* 0.87†
H_1-receptor antagonists		
Promethazine	3.0	0.030‡
Cyproheptadine	5.7	0.037§
Antidepressants		
Chlorimipramine	>10	0.053‡
Imipramine	>10	0.24† 0.14‡
Desmethylinipramine	>10	0.28‡
Amitriptyline	3.5	0.049* 0.036†
Mianserin	10.0	0.065*
Miscellaneous		
Chlorpromazine	5.9	0.041‡
Haloperidol	>10	0.081‡
Quinacrine	>10	0.26*

The antagonist potency on slices was determined against 1 μ M impromidine as described in Fig. 1 legend. Drugs were added 15 min before impromidine in at least five concentrations and IC_{50} values obtained by log-probit analysis of mean data from sextuplicate experiments. The EC_{50} mean value of impromidine in the absence of antagonist ($0.16 \pm 0.02 \mu$ M) was determined from seven different concentration-response curves. None of the drugs modified the basal level or interacted with the kinase binding assay. The K_i values for homogenates are from: *, ref. 24; †, ref. 18; ‡, ref. 19; §, ref. 17.

but having negligible potency on H_1 -receptors¹⁴. As shown in Fig. 2a, this compound stimulates cyclic AMP accumulation in hippocampal slices with an EC_{50} value of 0.17 μ M, that is, approximately 60 times lower than that of histamine and this effect is competitively antagonized by cimetidine, a H_2 -antihistamine, with a K_i value of 0.95 μ M similar to that found against histamine on peripheral H_2 -receptors¹³. Thus, both its relative potency and the inhibition constant of cimetidine indicate that H_2 -receptors mediate the stimulation elicited by impromidine. Promethazine antagonizes in a monophasic manner the response to impromidine with an IC_{50} of 22 μ M (Fig. 1). This antagonism is of the competitive type as shown by the parallel rightward shift of the concentration-response curve to this H_2 -agonist (Fig. 2a) and a K_i value of 6.2 μ M is thus obtained. This value closely agrees with that (5.5 μ M) obtained from the IC_{50} value regarding the second component of the response to histamine (Fig. 1). Again, as shown in Fig. 2b, a closely similar K_i value for promethazine (5.2 μ M) is obtained regarding its apparently competitive antagonism of the response to dimaprit, another selective agonist of H_2 -receptors possessing approximately 20% of the potency of histamine on peripheral biological systems¹⁵.

Hence, the almost identical K_i values of promethazine regarding inhibition of the responses to histamine and to two H_2 -agonists differing largely in chemical structure as well as in relative potency strongly support the conclusion that this compound has H_2 -antihistamine activity. However, it is important to notice that its potency in the guinea pig hippocampal slice preparation is approximately 100-fold lower than on the histamine-sensitive adenylyl cyclase of the cell-free preparation from the same region of guinea pig brain^{19,24}.

It was, therefore, of interest to determine the potency of other drugs that have been shown to strongly inhibit the cyclase in the cell-free preparation^{18,19,24}. Most of the compounds listed in Table 1, including H_1 -antihistamines, tricyclic antidepressants, antipsychotics and quinacrine, an inhibitor of histamine-N-methyltransferase, are significantly less potent on intact cells than on homogenates, the difference being, in most cases, more

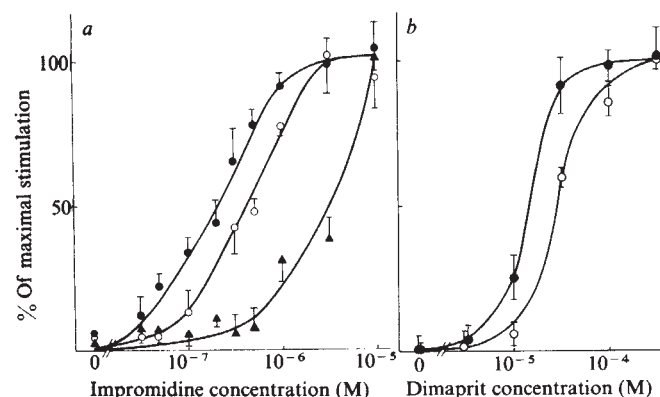


Fig. 2 Inhibition by promethazine or cimetidine of the stimulation of cyclic AMP accumulation in hippocampal slices elicited by two histamine H_2 -receptor agonists. Promethazine and cimetidine were added 15 min before the agonists and incubations performed as described in Fig. 1 legend. Results are expressed as the per cent of maximal stimulation elicited in the absence of antagonist, that is, (in pmol of cyclic AMP per mg protein) 17.5 ± 1.1 for impromidine (a) and 16.2 ± 0.5 for dimaprit (b), the mean basal level being 5.9 ± 0.4 . Mean $\pm$ s.e.m. of data from 3–6 values. The apparent dissociation constants of cimetidine, and promethazine were calculated according to the equation $K_i = A/(EC_{50}/EC_{50} - 1)$ where A is the concentration of antagonist, and EC_{50} and EC'_{50} the concentrations of agonist required to give half-maximal response in the absence or in the presence of antagonist. The K_i values for cimetidine and promethazine inhibiting the response to impromidine were $0.95 \mu M$ and $6.2 \mu M$, respectively. The K_i value for promethazine inhibiting the response to dimaprit was $5.2 \mu M$. ●, Control; ○, promethazine ($10 \mu M$ in a, $8 \mu M$ in b); ▲, cimetidine ($5 \mu M$).

than two orders of magnitude. However, this difference does not extend to the two H_2 -antihistamines, metiamide and cimetidine, which have a similar effect on the two systems, with apparent dissociation constants in close agreement with those (0.75 – $0.96 \mu M$) regarding inhibition of histamine effects on biological systems on which H_2 -receptors have been initially defined, like the increase of contraction of guinea pig atrium or the inhibition of electrically induced contractions of rat uterus^{13,25}. Hence it seems that the response to histamine on both systems can be considered to be mediated by H_2 -receptors as in both cases several H_2 -agonists exhibit the expected relative potencies and H_2 -antagonists the expected affinities. However, compounds like the tricyclic antidepressants clearly discriminate between the ' H_2 -receptors' from the two preparations, being much more potent than cimetidine on homogenates but less potent than this compound on intact brain cells.

The reasons for this discrepancy are not entirely clear at present but it is unlikely that the lower potency of compounds like promethazine or tricyclic antidepressants on the slice preparation results from their restricted diffusion into the intact tissue, for several reasons. First, their antagonist potency has been always determined following a 15-min period of preincubation to allow penetration into slices before the addition of histamine or agonists. Furthermore, we have observed for at least one compound, promethazine, that its potency was the same when it was added at the same time as histamine or impromidine. Second, promethazine has also a low potency (K_i of about $10 \mu M$, as in hippocampal slices) regarding blockage of H_2 -receptors mediating cyclic AMP accumulation in intact but isolated cells from stomach²⁶ or blood²⁷, that is, on preparations for which there is no diffusion problem. Indeed, as previously shown²², the affinity of promethazine is also lower for H_1 -receptors in slices compared to guinea pig ileum or 3H -mepyramine binding sites, suggesting some nonspecific mechanism leading to a decreased potency of drugs at different receptor types in this preparation. But this is not the case for cypro-

heptadine which inhibits the response due to H_1 -receptors in slices with an affinity (K_i , $0.005 \mu M$) closely comparable to that found in other preparations⁷ and nevertheless is 150 times less potent on H_2 -receptors from slices than from homogenates.

It could be argued that, being highly lipophilic molecules, the relatively low potency of central nervous system (CNS)-active compounds like promethazine on the slice preparation might be related to extensive fixation on to 'sites of loss' like lipids. This seems unlikely because the concentration of 3H -promethazine in the supernatant at the end of incubation is unchanged (data not shown).

It is also unlikely that the largely different potencies of these compounds on the two preparations reflect the interaction with two distinct subclasses of H_2 -receptors. Thus the cyclic AMP response to histamine in homogenates parallels that in slices in various brain regions: for instance in the guinea pig (one of the few responsive species with both preparations) it is observed only in three regions which exhibit the same order of amplitudes (hippocampus > neocortex > striatum) for both preparations^{16,28}.

It seems more likely that the distinct pharmacology of H_2 -receptors, when studied on a cell-free preparation as compared to intact cells, reflects modified drug discriminatory characteristics of these receptors which could be introduced either by the trauma of cell disruption or by the conditions required for optimal assay of adenylate cyclase activity. Several data already suggest that H_2 -receptors on this test system may differ from those mediating physiological responses to histamine in intact organs, even when these responses may be triggered by cyclic AMP acting as second messenger. Hence promethazine and tricyclic antidepressants exhibit, as in brain, high affinities for the histamine-activated adenylate cyclase of homogenates from guinea pig heart^{29,30} whereas both classes of agents seem to have inconsistent effects on the positive chronotropic and inotropic actions of histamine mediated by H_2 -receptors in the intact organ^{31,32}.

One could postulate that the histamine-binding portion of H_2 -receptors adopts a modified conformation when membranes which bear them are disrupted or when they are incubated in the presence of compounds like ATP, Mg^{2+} , GTP or EGTA which are required for optimal activation of the enzyme³³. Selective modifications of the drug specificity of several classes of membrane receptors in the presence of guanylnucleotides or metal ions have already been evidenced in the last years by radioligand binding methods^{34–36}. Alternatively, in the disrupted cell preparation some compounds might have access to regulatory or coupling units of the receptors that are normally hidden to them in the intact cell and thereby become potent inhibitors. Although this possibility seems *a priori* incompatible with the apparently competitive nature of the inhibition, established for most compounds^{18,19,24}, it cannot be entirely excluded in view of the complex interactions of histamine with the postulated allosteric sites for Mg^{2+} or nucleotides³³. These various hypotheses may be checked when 3H -ligands are available for studies of H_2 -receptors.

Whatever the mechanism involved, note that a large difference in pharmacological specificity of striatal dopamine receptors mediating the stimulation of cyclic AMP formation in slices and homogenates, respectively, has recently been observed³⁷, whereas this does not seem to be the case of β -adrenergic receptors³⁸. This suggests that the drug specificity of various types of receptors in cell-free systems has to be carefully compared to that in intact cell systems and that the idea that blockade of histamine H_2 -receptors represents the common molecular basis of clinical activity of tricyclic antidepressants requires confirmation by other approaches.

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Induction of experimental allergic encephalomyelitis in genetically resistant strains of mice

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Experimental allergic encephalomyelitis (EAE) is an acute autoimmune disease of the central nervous system. It is elicited in various laboratory animal species by a single injection of either whole central nervous system tissue homogenate, or the purified basic encephalitogenic protein isolated therefrom. Clinical manifestations of hind leg paralysis usually occur 11-21 days after challenge, preceded by histological lesions of perivascular infiltration in the brain¹. EAE has been intensively investigated both as a model for human demyelinating diseases and as a prototypic organ-specific autoimmune disease¹. EAE is under genetic control in guinea pigs, rats and mice, and strains that are genetically susceptible or resistant to EAE have been demonstrated²⁻⁴. We have previously^{5,6} investigated the genetic control of susceptibility to EAE in mice and demonstrated that the disease could be induced only in the SJL/J strain of mice, or in its crosses with various resistant strains. The resistance to EAE is not associated with genetic differences in the response of the various strains to pertussis vaccine, which is used as adjuvant in the induction of EAE in mice, as both the sensitive SJL/J strain and the resistant strains BALB/c, DBA/2 and C57BL/6J are responsive to pertussis⁷. A great difference was observed, however, in the sensitivity to EAE of the various F₁ hybrids of SJL/J. Thus, crossing the sensitive SJL/J strain with part of the resistant strains, such as NZB, BALB/c or its congenic strains BALB.B10 and BALB.C3H, led to fully susceptible hybrids, with even higher incidence of EAE than that observed in the parental SJL/J strain. On the other hand, crossing SJL/J with other strains, such as C57BL/6J or DBA/2, led to hybrids with very low susceptibility to EAE. These results suggested that in the crosses with the various resistant BALB strains and NZB strain, there is probably complementation with another gene. It was therefore postulated that in these resistant strains, the influence of the gene determining susceptibility to EAE is masked by the presence of naturally occurring suppressor cells. We present here experimental evidence which corroborates this assumption.

Mice of the resistant strain BALB/c were treated with various doses of cyclophosphamide (CY) 2 days before challenge with the basic protein (BE). This treatment, if carried out with

appropriate low doses of CY, was previously shown to eliminate selectively suppressor cell population in several systems, resulting in depression of suppressive cell-mediated activity and augmentation of both cellular and humoral immune responses⁸⁻¹¹. Figure 1 depicts the effect of CY on the susceptibility of BALB/c mice to the induction of EAE, showing that CY had a marked effect which is dose-dependent. At low doses (20 mg per kg), it drastically affected the resistance of the mice

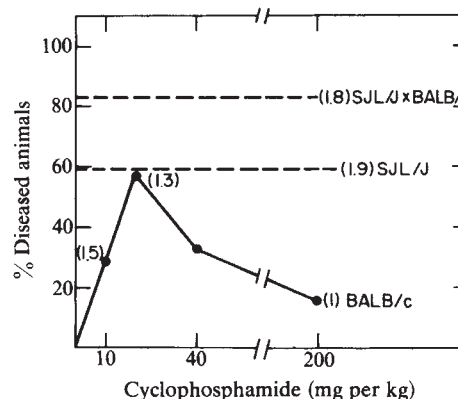


Fig. 1 Dose-response effect of CY on EAE induction in resistant BALB/c mice. EAE was induced by injecting 5 mg of dry lyophilized mouse spinal cord homogenate emulsified in 1:1 ratio in complete Freund's adjuvant supplemented with 4 mg ml⁻¹ of *Mycobacterium tuberculosis* (H₃₇Ra) (Difco). The emulsion in total volume of 0.12 ml was injected in all four footpads. Immediately after and 48 h later, pertussis vaccine (*Bordetella pertussis*, Rafa Laboratories) 10 × 10⁹ organisms, were injected intravenously. Mice were examined daily from day 11 post-induction for signs of EAE. Signs of disease were scored from 1 to 3 according to the clinical situation as follows: 1, loss of weight and loss of tail tonicity; 2, mild disease: paresis and paralysis of hind legs; 3, severe paralysis often leading to death. Animals were killed 18-24 days after injection and the whole brain and spinal cord were taken into 10% formalin solution for histological examination. Sagittal sections of brains and spinal cords, fixed in formalin and stained with Luxol fast blue, were examined microscopically for perivascular infiltration of mononuclear cells. The observations were done under code without knowledge of the injection regime. Solutions of CY (Polysciences), were freshly prepared by dissolving in minimal volume of ethanol and then diluted 100-fold in phosphate-buffered saline. CY was injected intraperitoneally 2 days before EAE induction. The numbers in the parentheses represent the mean score of disease signs. The broken lines represent the incidence of EAE in SJL/J and SJL/J × BALB/c mice without CY.

to EAE—in these conditions BALB/c mice succumbed to the disease to the same extent and with the same severity as the susceptible strain SJL/J. Lower or higher doses of CY were less effective. High doses of CY (>200 mg per kg) affect the whole lymphoid system and suppress EAE induction altogether¹².

The effect of CY at its most effective concentration (20 mg per kg) was tested in additional resistant strains of various H-2 types. The results summarized in Table 1 demonstrate that in BALB.B10 and BALB.C3H, the congenic strains of BALB/c, and to a lesser extent in the NZB strain, CY had a similar effect on the susceptibility to EAE to that demonstrated in BALB/c. On the other hand, in the other strains, such as C57BL/6J, DBA/2, AKR/J, ASW, B10.A and A/J, CY had no effect on the development of EAE. This phenomenon is therefore not linked to the H-2 type. It should be pointed out that in two of the these strains, C57BL/6J and B10.A, we have tested the effect of different doses of CY. No effect on EAE induction was observed at doses of 10, 20, 40 or 200 mg per kg. This experiment rules out the possibility that a non-optimal dose of CY was the factor responsible for the persistent unresponsiveness to EAE in those strains, and strengthens the conclusion that the involvement of suppressor cells in the resistance to EAE is not H-2 linked.

Similar effects to those demonstrated by CY on suppressor cells were also demonstrated in other systems by other immunosuppressive drugs, antilymphocyte serum, as well as by low-dose irradiation^{8,11}. We therefore tested whether a similar effect to that demonstrated for CY on EAE induction could also be achieved using irradiation. BALB/c mice were exposed to whole body low-dose irradiation (200 R and 350 R) at various times before EAE induction. The results summarized in Table 2 demonstrate that when the mice were irradiated with 350 R 2 days before the encephalitogenic challenge, 50% of the otherwise resistant mice developed EAE.

It thus seems that there are two types of inbred strains that are resistant to EAE. Treatment with CY or low-dose irradiation can convert one type from resistant into susceptible, while it has no effect on the other type. It is suggested that in strains of the first type, a gene that is responsible for susceptibility to EAE does exist, but these strains probably maintain a natural high level of suppressor cells which prevent the manifestations of disease. Following treatment with CY, these suppressor cells are eliminated and disease is overt. In the F₁ hybrid, the balance between effector cells and suppressor cells is shifted towards the effector cell function, and therefore EAE can be induced. In

Table 2 Effect of irradiation on EAE induction in BALB/c mice

Treatment	Mice with EAE	
	Clinical	Histological
None	0/7	0/7
200 R on day -2	0/7	NT
350 R on day -2	5/10	8/10
200 R on day 0	1/10	NT
350 R on day 0	3/10	3/6

NT, not tested.

addition, there is complementation of genes from the two parents and the resultant F₁ may be even more sensitive. In rare cases, some animals of such strain may possess a lower level of suppressor cell, leading to the very low incidence of EAE in three resistant strains, even without CY treatment. In the second type of resistant strains, such as DBA/2, there is no Ir gene for EAE and therefore CY has no effect.

The results with CY and low-dose irradiation, although suggesting involvement of suppressor cells in regulation of sensitivity to EAE, do not provide unequivocal proof of the existence of such cells in resistant strains. However, we have recently demonstrated that CY eliminates suppressor cells involved in antigen-induced unresponsiveness to EAE¹³. In that study, protection against EAE¹⁴ was induced in susceptible mice (SJL/J × BALB/c hybrid) by injection of an adequate protective antigen (spinal cord homogenate, BE or the synthetic polymer Cop 1) in incomplete Freund's adjuvant, before EAE induction. This unresponsiveness could be adoptively transferred by spleen T lymphocytes to normal syngeneic recipients. Low dose of CY (20 mk per kg) abrogated both actively induced and adoptively transferred unresponsiveness to EAE. It thus appears that in that system as well, the treatment with CY results in the elimination of suppressor cells, which interfere with the effector mechanisms responsible for EAE.

It is now well established that suppressor cells have a critical role in immune regulation, including maintenance of tolerance, antigenic competition and genetic control of immune responses¹⁵. It was also suggested that specific T-cell-mediated suppression could be one of the physiological processes of immune responsiveness against autoantigens¹⁶. In NZB mice it has been demonstrated that loss of effective suppressive T cell function can lead to autoimmune diseases¹⁷. The use of selective means for elimination of suppressor cells may assist in the elucidation of such activity in autoimmunity. Indeed, it was demonstrated that CY can induce a transient appearance of 'autoreactive' T lymphocytes in spleens of mice¹⁸. This accumulated information corroborates our present conclusion suggesting suppressor cell involvement in both natural and induced resistance to EAE.

Table 1 Effect of low-dose CY on EAE induction

	H-2 type	Mice with EAE					
		Without CY			With CY		
		Clinical	Mean score	Histological	Clinical	Mean score	Histological
SJL/J	s	10/16	2.0	10/10	7/16	1.8	6/6
(SJL/J × BALB/c)F ₁	s/d	18/20	1.8	20/20	20/22	2.2	10/10
BALB/c	d	0/40		1/30	19/32	1.7	13/20
BALB.B10	b	1/28	1.0	1/5	4/11	1.3	4/10
BALB.C3H	k	2/20	2.0	1/11	4/8	1.8	4/8
C57BL/6J	b	1/19	1.0	2/19	0/18		1/8
NZB	d	0/7		0/5	2/8	2.0	5/7
DBA/2	d	0/6		0/6	0/5		0/5
AKR/J	k	0/19		0/9	0/17		0/8
B10A	a	0/7		0/7	0/9		1/8
ASW	s	0/11		2/6	0/10		0/7
A/J	a	0/6		0/6	0/6		0/4

EAE was induced as described in Fig. 1 legend. CY (20 mg per kg) was injected 2 days before disease induction.

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Hormonal control of neurotransmitter choice in sympathetic neurone cultures

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Manipulation of the cellular and hormonal environment of cultures of dissociated primary neurones can be used to explore a neurone's developmental potential and to investigate the factors required for normal development. For example, developing adrenergic sympathetic neurones can be influenced to become cholinergic by both diffusible¹ and membrane-bound² factors from certain types of non-neuronal cells; when medium conditioned by incubation on heart cell cultures (CM) is placed on the neurones, they develop the ability to produce acetylcholine (ACh) and they form functional cholinergic synapses with each other³. Hormones could also contribute to the control of this transmitter choice⁴, and McLennan *et al.*⁵ recently reported that corticosterone treatment of whole superior cervical ganglia (SCG) greatly inhibited the cholinergic development of these ganglia in culture. It was not clear, however, whether the hormone acted directly on the neurones or indirectly via the non-neuronal cells. To study the role of hormones on this transmitter choice, I have now developed a serum-free medium (based on that of Sato and co-workers^{6,7}) for the preparation of conditioned medium. The results obtained with this system show that glucocorticoids and epidermal growth factor (EGF) exert dramatic and antagonistic effects on the adrenergic-cholinergic transmitter choice and do so indirectly, by controlling the ability of heart cells to produce cholinergic CM.

Heart myocytes and heart fibroblasts (both simply called 'heart cells') were obtained by collagenase treatment of whole hearts from neonatal rats and grown in flasks containing 10% fetal calf serum (FCS, MA Bioproducts) in L-15 CO₂ medium¹. After the cells had grown to confluency, they were either used for conditioning or were trypsinized and replated to be used as secondary cultures. To obtain conditioned medium, the flasks were washed with serum-free medium and incubated for 24 h with the medium to be conditioned. The CM was collected and frozen at -20 °C and the flasks were incubated for 24 h with the 10% FCS medium again. After washing, a second batch of medium to be conditioned was placed in the flasks for 24 h. This CM was collected and also frozen. Before addition to the neuronal cultures, the CM was thawed and mixed with an equal volume of fresh L-15 CO₂ medium containing, in addition to the usual components of the neuronal growth medium⁸, 10% rat serum and 3 mg ml⁻¹ Methocel. Control neuronal cultures received medium containing 5% rat serum and 1.5 mg ml⁻¹ Methocel. In both cases, the medium was changed every 2 days. The neurones were mechanically dissociated from neonatal rat SCG and plated as previously described⁸. All neuronal cultures received control medium for the first 2 days and then experimental media until days 14–18, when they were assayed. Ganglionic non-neuronal cells were killed by treatment with cytosine arabinoside⁸. ³H-ACh synthesis from ³H-choline and ³H-catecholamine (CA) synthesis from ³H-tyrosine were assayed as previously described^{1,9}. The ratio of synthesis of these transmitters, ACh/CA, is a sensitive indicator of the number of cholinergic as opposed to adrenergic neurones in this culture system¹⁰.

Heart cells were grown with various combinations of hormones and growth factors as substitutes for serum. CM from these cultures were tested for induction of ACh synthesis in neuronal cultures. The results of a typical experiment testing a variety of hormones are shown in Table 1a. CM from heart cells

Table 1 Effects of hormones on cholinergic induction

Additives	ACh/CA	ACh (pmol per dish)	CA (pmol per dish)
a BSA	0.03 ± 0.01	0.20	7.26
EGF	0.18 ± 0.03	1.22	6.53
EGF + BSA	2.47 ± 0.10	12.53	5.07
EGF + BSA + insulin	7.53 ± 0.71	25.25	3.38
EGF + BSA			
+transferrin	2.00 ± 0.09	13.91	6.99
EGF + BSA + insulin			
+transferrin	9.09 ± 0.15	32.86	3.62
EGF + insulin			
+transferrin	0.26 ± 0.01	2.48	9.73
BSA + insulin			
+transferrin	0.04 ± 0.01	0.51	11.98
Insulin + transferrin	0.04 ± 0.01	0.44	11.12
b 5% Rat serum	4.20 ± 0.09	18.56	4.41
5% Rat serum			
+hydrocortisone	1.57 ± 0.12	8.40	5.33
EGF + BSA + insulin			
+transferrin	9.09 ± 0.15	32.86	3.62
EGF + BSA + insulin			
+transferrin			
+hydrocortisone	0.62 ± 0.12	9.85	15.60
5% Rat serum + EGF			
+insulin + transferrin	6.00 ± 0.88	24.14	4.08
5% Rat serum + EGF			
+insulin + transferrin			
+hydrocortisone	4.92 ± 0.40	24.67	4.94

Confluent heart cells were incubated in L-15 CO₂ medium with various combinations of hormones and growth factors as substitutes for serum. The concentrations used were: BSA, 2.5 mg ml⁻¹ (Sigma); EGF, 20 ng ml⁻¹ (purified according to Savage and Cohen¹⁵ or from Collaborative Research); hydrocortisone, 10⁻⁶ M (Sigma); insulin, 5 µg ml⁻¹ (Sigma); selenium, 30 nM (Johnson Matthey, treated as in ref. 14); transferrin, 25 µg ml⁻¹ (Sigma). Sympathetic neurones were grown in 50% CM from these cultures and transmitter synthesis was assayed on day 18.

Table 2 Identification of target of EGF action

	ACh/CA	ACh (pmol per dish)	CA (pmol per dish)
a Complete serum-free medium incubated in an empty flask	0.05 ± 0.01	0.17	3.26
b Complete serum-free medium incubated on heart cells	3.66 ± 0.25	12.57	3.44
c Serum-free medium, without hormones, incubated on heart cells, with fresh hormones added before placing on neurones	0.05 ± 0.01	0.25	4.70
d Complete serum-free medium (-EGF) incubated on heart cells, with fresh EGF added before placing on neurones	0.05 ± 0.01	0.19	3.90

Sympathetic neurones were grown for 14 days in 50% CM made by incubation in conditions a–d.

treated with medium containing bovine serum albumin (BSA) as the only additive gave the usual low ACh/CA ratio expected for medium with no additions at all. If EGF was used as the only additive, there was a significant increase in the ACh/CA ratio. This effect of EGF was greatly enhanced by the addition of BSA. Similarly, insulin had a synergistic effect on the transmitter ratio

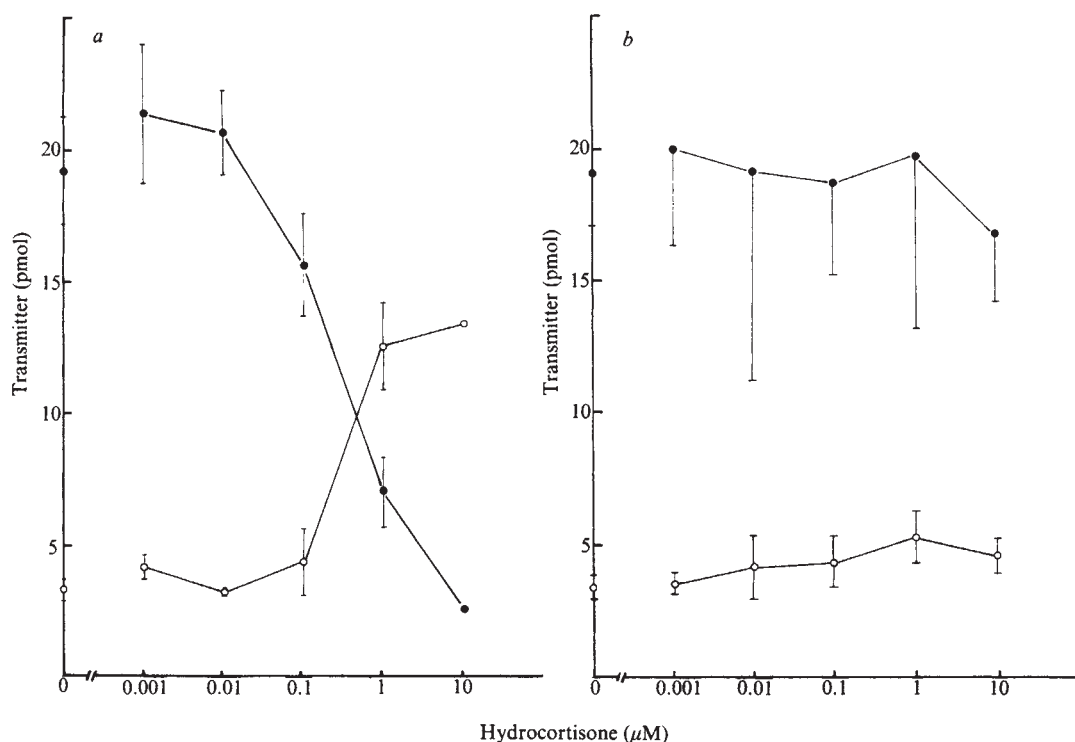


Fig. 1 *a*, Hydrocortisone was added at various concentrations to heart cells in complete serum-free medium for 24 h and the CM was tested for induction of neuronal ACh synthesis. *b*, Fresh hydrocortisone was added directly to neurones in complete serum-free medium conditioned on heart cells. The amounts of transmitters synthesized at day 17 (●, acetylcholine; ○, catecholamine) are expressed as pmol per dish. Points are means $\pm$ s.e.m. of triplicate cultures.

when added along with EGF, but had no effect in the absence of EGF. The effect of transferrin was variable and small. The most potent CM was consistently obtained using EGF, BSA, insulin and transferrin, and medium containing these additives is termed 'complete' serum-free medium.

In comparison, hydrocortisone had a striking inhibitory effect on the transmitter ratio. As shown in Table 1*b*, this hormone antagonizes the cholinergic effects of both the complete serum-free CM and CM containing the usual 5% rat serum. The 15-fold effect of hydrocortisone on the transmitter ratio in serum-free conditions is typical of the values seen in more than 10 such experiments. Other glucocorticoids (corticosterone, cortisone and dexamethasone) showed similar antagonistic effects on ACh induction. Both the cholinergic effect of complete serum-free conditioned medium and the antagonistic effect of hydrocortisone are reduced in the presence of 5% rat serum, suggesting that the complex mixture of hormones in serum obscures the effect of individual hormones.

Other hormones which showed small positive effects on the transmitter ratio were fibroblast growth factor (100 ng ml⁻¹, Collaborative Research) and relaxin (10 ng ml⁻¹) when added together with BSA, insulin and transferrin. Hormones showing little effect on the ratio were growth hormone (20 ng ml⁻¹), 3,3',5-triiodothyronine (10⁻¹⁰ M, Sigma), prolactin (10 ng ml⁻¹), partially purified thyroid stimulating hormone (100 ng ml⁻¹) and follicle stimulating hormone (0.5 $\mu\text{g ml}^{-1}$), progesterone (10⁻⁹ M, Sigma), testosterone (5 $\times$ 10⁻⁹ M, Sigma), oestradiol (5 $\times$ 10⁻⁹ M, Sigma) and aldosterone (10⁻⁹ M, Sigma). Selenium, which is essential in some serum-free systems⁷, had little effect on the transmitter ratio.

Do the hormones influence transmitter choice by acting directly on the neurones, or indirectly, by altering the ability of the heart cells to produce cholinergic CM? These possibilities were tested in the experiments shown in Table 2 and Fig. 1. Complete serum-free medium was incubated for 24 h in either an empty flask (*a*) or in one containing heart cells (*b*), and these CM were tested on the neurones. As expected, the heart cells were essential for the induction. Furthermore, if the hormones were added to serum-free conditioned medium (minus hormones, incubated on the heart cells) immediately before it was placed on the neurones (*c*), no cholinergic induction occurred. Addition of fresh EGF to complete (except for EGF) serum-free CM was similarly ineffective when added directly to

the neurones (*d*). EGF had a very striking cholinergic inductive effect when added at 10 ng ml⁻¹ on the heart cells, but had no effect when added directly to the neurones, even at higher concentrations (data not shown). Thus, the EGF effect on neuronal transmitter choice is an indirect one, via the heart cells.

When the same type of experiment was conducted with hydrocortisone, two different effects were seen (Fig. 1). When added to the heart cultures (Fig. 1*a*), this hormone antagonized the cholinergic effect of the complete serum-free CM, resulting in both lower ACh and higher CA production by the neurones. On the other hand, addition of hydrocortisone to the CM just before placing it on the neurones had little effect on cholinergic development, but may have slightly increased CA production (Fig. 1*b*). This latter effect has been observed before in this system (L. Chun, P. Walicke & P. H. Patterson, unpublished

Table 3 Effects of hormones on heart cells

	Labelled protein (c.p.m.)	Total protein (μg per dish)
<i>a</i> No hormones	7,951 $\pm$ 243	0.23 $\pm$ 0.04
EGF + BSA + insulin		
+ transferrin	11,620 $\pm$ 448	0.26 $\pm$ 0.01
EGF + BSA + insulin		
+ transferrin + hydrocortisone	10,732 $\pm$ 110	0.27 $\pm$ 0.04
5% Rat serum	12,850 $\pm$ 2,095	0.28 $\pm$ 0.01
5% Rat serum + hydrocortisone	13,708 $\pm$ 1,288	0.27 $\pm$ 0.01
		DNA (μg per flask)
<i>b</i> BSA + insulin + transferrin		26.2
EGF + BSA + insulin + transferrin		26.6
EGF + BSA + insulin + transferrin		
+ hydrocortisone		30.7
5% Rat serum		34.3
5% Rat serum + hydrocortisone		32.9

a, Confluent heart cells were labelled with ³H-leucine in L-15 CO₂ medium supplemented with hormones as indicated. After a 24-h incubation, the cells were washed with several changes of unlabelled medium for 30 min and then lysed with 2% SDS. The lysate was used for measuring both synthesized protein (total counts) and total protein (by Lowry's method¹⁵). *b*, The DNA content of heart cells in a similar experiment as in Table 1 was determined by pentose analysis¹⁶.

results) and could be due to a NGF-mediated induction of tyrosine hydroxylase and dopamine- β -hydroxylase¹¹. Thus, the dramatic effect of hydrocortisone on transmitter choice, like that of EGF, is primarily an indirect one, via the non-neuronal cells. Note that the concentrations of glucocorticoids which were effective in these experiments (10^{-7} – 10^{-5} M) are similar to the plasma corticosterone level in neonatal rats (10^{-6} M)¹².

These results raise the question of whether the hormonal actions are due to nonspecific effects on growth or health of the heart cells. At the crudest level, EGF and hydrocortisone could have stimulatory and deleterious effects, respectively, on the number or growth of the heart cells. While this may be the case over extended periods of incubation, the 24-h incubation with hormones used in these experiments results in little alteration in the total DNA or protein content of the heart cell cultures (Table 3). Could the hormones be having effects on overall heart cell protein synthesis which was not reflected in the total protein content after only 24 h of incubation? This seemed unlikely as the hormones had little effect on ³H-leucine incorporation into protein during the 24-h incubation (Table 3). Furthermore, in preliminary experiments, when ³H-leucine-labelled protein secreted into the medium by heart cells was examined by SDS-gel electrophoresis, there were no gross differences in the patterns due to the presence of EGF or hydrocortisone (data not shown). The hormones thus appeared to have no obvious stimulatory or deleterious effects on the growth or health of the heart cells or on major proteins secreted during the 24-h incubation.

The results presented here suggest a further level of subtlety in the control of neurotransmitter choice in sympathetic neurones: specific hormones can act on non-neuronal cells to

alter selectively the production or liberation of a factor (or factors) that induces neuronal cholinergic development and reduces adrenergic development.

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A neuropeptide in mammalian tissues with physalaemin-like immunoreactivity

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Ersparmer *et al.*^{1–3} isolated several structurally related non-mammalian peptides, termed tachykinins^{2,3}, with biological activities similar to substance P (refs 2, 3). This similarity is due to a common COOH-terminal region^{2,3}, whereas the specific action of each member of the group depends on its unique NH₂-terminal sequence^{3–6}. Based on these properties, parallel bioassays using numerous smooth muscle preparations and physiological parameters allow them to be generally distinguished^{2,3,6}. However bioassays are time consuming and not highly specific. Thus, we developed a rapid physalaemin-specific radioimmunoassay that led to the initial discovery of a substance in mammalian tissues with an immunoreactivity resembling that of the original amphibian peptide⁷. We now present data on the distribution, localization and partial characterization of physalaemin-like immunoreactivity (PSLI) in tissues of several mammalian species.

Details of the preparation of the antiserum and the radioimmunoassay are described elsewhere⁷. Antiserum (PS-1) had a titre of 1:40,000 and specifically recognized the NH₂-terminal region of physalaemin with only minimal cross-reactivity with the other tachykinins (Table 1). Other bioactive peptides had negligible cross-reactivity (<0.0001%)⁷. Antiserum PS-1 cross-reacted with uperolein $2.03 \pm 0.94\%$ (mean $\pm$ s.d., $n = 4$) (Table 1), but compared with physalaemin the displacement curve was nonparallel (the ratio of the slopes of physalaemin:uperolein =

2.10 ± 0.20 ; Fig. 1). The cross-reactivity of antiserum PS-1 using ¹²⁵I-uperolein yielded a complementary set of data: at a constant bound/free ratio (B/F) a lower antiserum dilution was required ($1:6,000$) and the sensitivity of the assay to physalaemin decreased fourfold to 29.8 ± 6.4 pg ($n = 4$) (Table 1); the cross-reactivity of physalaemin was also nonparallel relative to uperolein (ratio = 2.43 ± 0.88 , $n = 3$; Fig. 1); and 50% displacement by uperolein was 51.0 ± 14.2 pg (40.7 ± 11.3 fmol, $n = 4$; Fig. 1). As also shown in Fig. 1, a crude extract of guinea pig stomach and the peak material from either Bio-Gel P-4 or HPLC (Fig. 3) produced parallel displacement curves relative to the labelled peptide used. Thus, it appeared that PSLI in tissue extracts contained an amino acid sequence common to the NH₂-terminal region of both physalaemin and uperolein (-Asp-Pro-Asn-; Table 1). Predictably, the ratio between their slopes should be identical, and within experimental limitations this was the case. Although the concentration of immunoassayable substance would be higher by nearly an order of magnitude using ¹²⁵I-uperolein, PSLI was quantified on the basis of physalaemin as the antiserum was raised against this peptide.

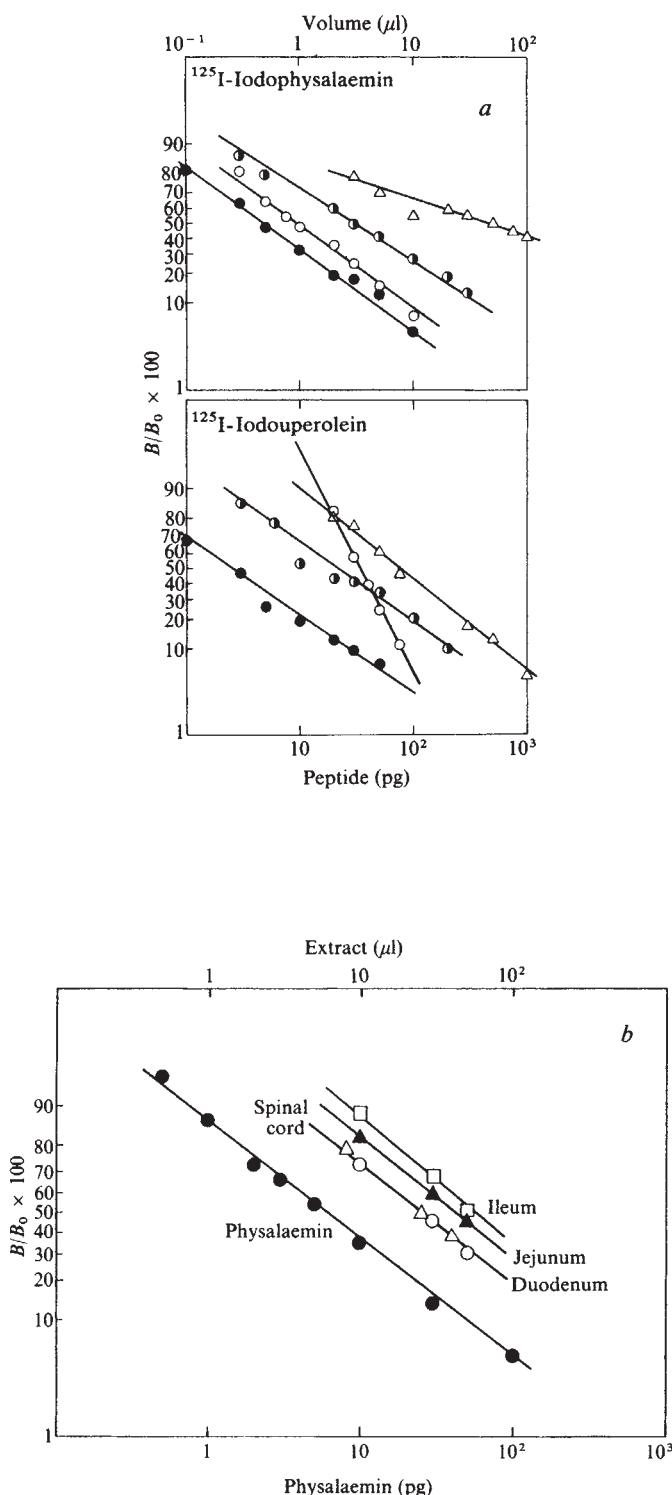
PSLI was found throughout the gastrointestinal tract of all the mammalian species examined and exhibited parallel displacement to physalaemin (Fig. 1). The actual concentration in each region of the stomach varied considerably between species (Table 2), with the rabbit containing the highest levels⁷. Guinea pig stomach contained more PSLI than any other region of the gastrointestinal tract: it was higher in the duodenum than in either the jejunum or ileum and a similar quantity was found there to that in the pylorus and fundus (Table 2). In rabbit, the level of PSLI in the duodenum was considerably less than that found in the stomach. PSLI was also found in porcine oesophagus in the mucosal and muscle layers, and mucosa of the trachea, as well as in rabbit trachea. Inconsistent values and nonparallel displacement curves, similar to that produced by proteases, frequently occurred with extracts of brain, lung and plasma from rat and guinea pig.

The large amounts of PSLI in the gastrointestinal tract may indicate a functional role for this material. The detection of relatively small quantities of PSLI in tissues containing a high

Table 1 Specificity of physalaemin antiserum PS-1 among the tachykinin group of polypeptides

Peptide	Sequence	Molar cross-reactivity (%)	
		¹²⁵ I-physalaemin	¹²⁵ I-uperolein
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂	100	174.0 ± 38.9
Uperolein	—Pro—Ala—	2.03 ± 0.94	100
Phyllomedusin	pGlu-Asn—Arg—	0.36 ± 0.04	0.32
Substance P	Arg-Pro-Lys—Gln-Gln—Phe—	<0.00013	0.05
Eledoisin	—Pro-Ser-Lys-Asp—Ala—Ile—	<0.0001	NT
Kassinin	Asp-Val-Pro-Lys-Ser-Asp—Glu—Val—	0.013 ± 0.003	NT

Cross-reactivity is derived from the 50% displacement values by unlabelled peptide and given as the mean ± s.d. The sensitivity of antiserum PS-1 for physalaemin using ¹²⁵I-physalaemin is 6.79 ± 1.98 pg (5.29 ± 1.54 fmol, *n* = 34) and for uperolein using ¹²⁵I-uperolein (at a titre of 1:6,000 final dilution) 51.0 ± 14.2 pg (40.7 ± 11.3 fmol, *n* = 4). NT, not tested.



density of nerve fibres, for example, the sphincter areas of the stomach⁸, may be masked by extraneous protein in the extract. On the other hand, subtle structural modifications may also affect its quantification: studies on amino acid variants of kassinin and physalaemin⁶, charge differences in luteinizing hormone releasing factor⁹ and vasoactive intestinal polypeptide (VIP)¹⁰, and NH₂-terminally extended bombesin¹¹ and somatostatin¹² support this possibility. However, relatively small amounts of PSLI could exert profound effects as physalaemin is one of the most potent pharmacologically active peptides known³.

Immunostaining was observed in both epithelial cells and nerve fibres in the gastrointestinal tract of guinea pig and rat. In guinea pig, the immunostaining can be summarized as follows: (1) scattered epithelial cells with PSLI in villi throughout the gastrointestinal tract; (2) the most conspicuous feature was the numerous, strongly immunostained cells in Brunner's glands¹³ of the proximal duodenum (Fig. 2a); (3) submucosal glands of the ileum; (4) beaded, fibre-like structure, probably representing terminal and preterminal nerves in Meissner's and Auerbach's plexi in the duodenum; and (5) beaded fibres with PSLI among pancreatic acinar cells that are also lightly immunostained. In rat, immunostained cells were also widely distributed throughout the gastrointestinal tract, but scattered immunostained epithelial cells were most frequent in the colon (Fig. 2b). Also in rat, beaded fibres with PSLI were observed in the muscle layers of the gastro-oesophageal sphincter and surrounding unstained ganglion cells of Auerbach's plexus in the same region (Fig. 2c). The localization of PSLI in epithelial cells and nerve fibres of the gastrointestinal wall resembled that of substance P (refs 14–16), neurotensin^{17–19}, motilin¹⁴, enkephalin^{16,20},

Fig. 1 a, Specificity of the physalaemin radioimmunoassay. The assay contained 50 mM sodium cacodylate, pH 5.75, 1.0 mg bovine serum albumin, 4,000–5,000 c.p.m. ¹²⁵I-physalaemin or ¹²⁵I-uperolein, antiserum PS-1 (final dilution 1:40,000 with ¹²⁵I-physalaemin and 1:6,000 with ¹²⁵I-uperolein) and sample or standard peptide in a total volume of 100 μl. After incubation at 2–4 °C for 16 h, nonimmune rabbit serum and goat anti-rabbit antiserum (Cappel) were added to final dilutions of 1:2,000 and 1:50, respectively. The assays were incubated for 3 h at 22 °C, diluted with 1.0 ml buffer, centrifuged (2,500g for 30 min) and the residue counted⁷. Control tubes lacking antiserum PS-1 or in the presence of 60 pmol physalaemin had <1.0% of the total radioactivity. The plots contain standard curves for physalaemin (●), uperolein (Δ), an extract of guinea pig stomach (○) and the peak material from either Bio-Gel P-4 column or HPLC (Fig. 3) (◐). The upper panel shows the assay containing ¹²⁵I-physalaemin whereas the assay in the lower panel contains ¹²⁵I-uperolein. b, Physalaemin-like immunoreactivity in extracts of guinea pig tissue. Lyophilized tissues were powdered, then extracted in 10 volumes (wet weight) 1 mM EDTA-1 mM dithiothreitol at 98 °C. After cooling, they were acidified (1.0 M formic acid), homogenized (Polytron) and centrifuged (27,000g for 15 min at 4 °C). The pellets were re-extracted and the combined supernatants lyophilized⁷. The residue was dissolved in 50 mM cacodylate buffer, pH 5.75, and clarified before use. The data from a typical experiment are presented as the per cent B/B₀ against pg physalaemin (●), or volume of extract from duodenum (○), jejunum (Δ), ileum (◐) or spinal cord (Δ). The maximum variation between the slopes of the lines relative to physalaemin was 3%.

Table 2 Physalaemin-like immunoreactivity in mammalian tissue

Tissue	Amount in lyophilized tissue* (ng g ⁻¹)	
<i>Guinea pig</i>		
Fundus	17.64 ± 3.77	
Corpus	5.87 ± 3.80	
Pylorus	11.10 ± 3.22	
Duodenum	14.86 ± 2.54	
Jejunum	4.26(5.26, 3.26)	
Ileum	2.69(3.72, 1.65)	
Caecum	3.17(2.64, 3.69)	
Spinal cord	1.08(1.43, 0.73)	
Urinary bladder	1.86(1.16, 2.55)	
Pancreas	2.74(2.11, 3.36)	
<i>Mouse</i> †		
Fundus	5.59	
Pylorus	6.69	
<i>Rat</i> ‡	Adult	Neonatal
Fundus	2.29 ± 0.68	3.89
Pylorus	5.53 ± 0.79	3.86
Small intestine	ND	2.45

ND, not determined.

* Dry weight constitutes 21.5 ± 2.8% of the wet weight. Values are mean ± s.d. (n = 3–4).

† These figures are from four Swiss-Webster mice.

‡ Data are from five adult male CD-2 rats and seven neonatal pups killed at 24 h postpartum.

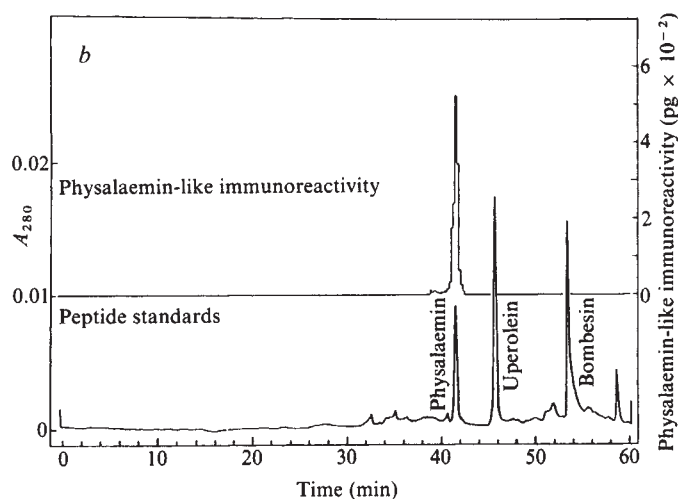
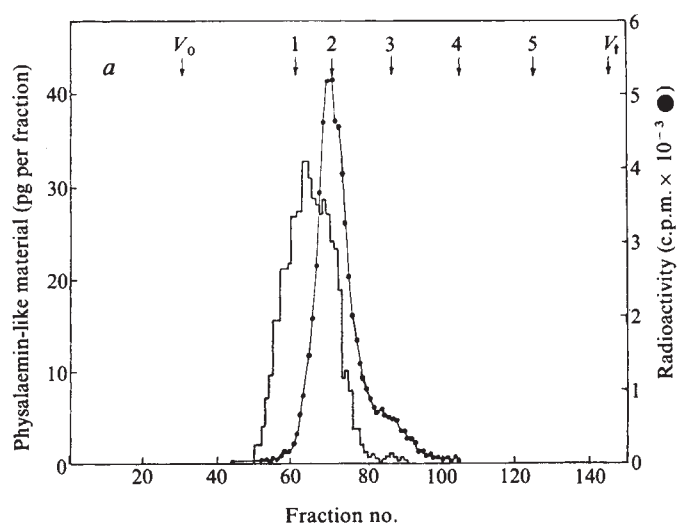


Fig. 3 *a*, Column chromatography of extracts of guinea pig stomach. Bio-Gel P-4 (100–200 mesh; Bio-Rad) columns (0.7 × 48.5 cm) were equilibrated and eluted with 0.1 M formic acid. Fractions (0.23 ml) were collected at a flow rate of 10.4 ml h⁻¹ at a hydrostatic pressure of 100 cm. Each fraction was lyophilized and redissolved in 0.15–0.4 ml water or cacodylate buffer before assaying. Immunoreactivity (bar graph) and ¹²⁵I-iodophysalaemin (●) are presented. A physalaemin standard (determined by radioimmunoassay) eluted in the same fractions as iodinated physalaemin due to variability in column chromatography: the s.d. in the peak is ±1.7 fractions⁷. The arrows indicate the position of the standards: V₀, Bovine serum albumin, 1, [¹²⁵I-Tyr⁸]-bombesin; 2, [¹²⁵I]-physalaemin; 3, [¹²⁵I-Des-Gly⁹-Met-NH₂¹¹]-physalaemin; 4, [¹²⁵I-des-pGlu¹-Phe⁷]-physalaemin; 5, [¹²⁵I-Tyr-Phe]; and V_t, [¹²⁵I-Tyr or DNS-Ala. *b*, HPLC fractionation of the PSII peak material from Bio-Gel P-4 columns. Fractions 55–75 were pooled, lyophilized, and the samples (100 μl) were fractionated on a Particil 5 ODS reverse-phase column (0.45 × 25 cm) (Whatman) in 10 mM Tris-phosphate, pH 7.05, and 20% methanol. Elution was carried out with a 20–65% linear methanol gradient in 45 min after an initial delay of 5 min at a flow rate of 0.8 ml min⁻¹ (O.H. *et al.*, in preparation). Fractions were collected at either 0.1- or 0.2-min intervals, the methanol evaporated under N₂, lyophilized and dissolved in buffer. The lower tracing is the absorbance of a mixture containing 6 μg of physalaemin, uperolein and bombesin; the retention times of each were determined independently. The bar graph in the upper curve indicates PSII.

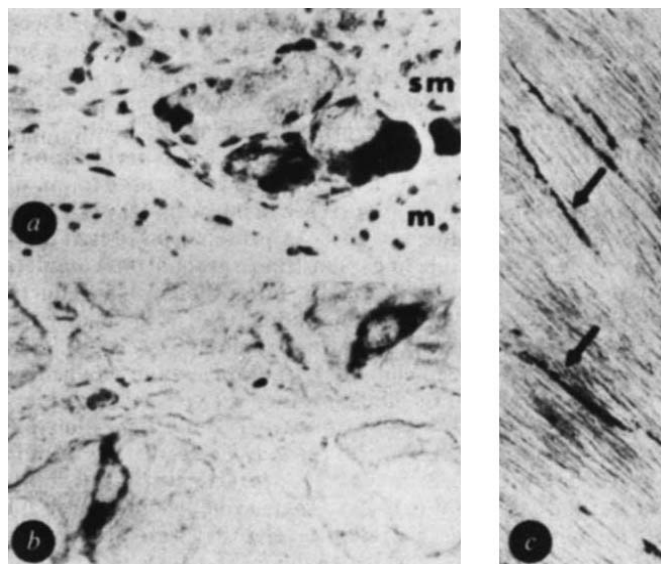


Fig. 2 Immunocytochemical localization of physalaemin-like immunoreactivity in the gastrointestinal tract. Immunostaining was carried out using the immunoperoxidase bridge technique³⁵. The best results were obtained by freezing the tissue in 2-methylbutane at the temperature of liquid nitrogen, freeze-drying and fixing in the vapours of diethylpyrocarbonate³⁶. Antiserum PS-1 gave satisfactory staining at a dilution of 1:1,000. Immunostaining was eliminated with the incubation of serial sections with either normal rabbit serum or antiserum PS-1 that was preadsorbed with 100 μg of physalaemin antigen. Immunostaining was not affected by preadsorption with 100 μg substance P or uperolein. *a*, Brunner's gland in the guinea pig duodenum showing dark, homogeneous staining (on the right side) in selected cells in the gland; sm = submucosa, m = muscle (circular layer). ×290. *b*, Two epithelial cells in rat colon; note that the nuclei are unstained. ×730. *c*, Beaded nerve fibres (arrows) with PSII among the smooth muscle fibres of the gastro-oesophageal sphincter of the rat. ×290.

somatostatin^{14,16}, VIP (refs 8, 13, 21, 22), bombesin^{13,23,24} and cholecystokinin²⁵, suggesting that the mammalian form of physalaemin may belong to this diverse group of neuropeptides.

Column chromatography of extracts of guinea pig stomach on Bio-Gel P-4 columns ($n = 15$) gave only one symmetrical peak of PSLI (molecular weight 1,700) regardless of the area of the stomach examined. This was larger than any of the known tachykinins⁶ and no other major peak with PSLI eluted from the columns. Cyanogen bromide digestion of the peak material gave an immunoreactive profile with the same elution volume as that of the original extract, suggesting that PSLI could be NH₂-terminally extended. This indeed was found for a mammalian bombesin-containing peptide¹¹, angiotensin from frogs⁵ and somatostatin from porcine intestine¹². Reverse-phase HPLC was used to resolve whether PSLI resembled physalaemin or uperolein: in acidic or neutral conditions, PSLI co-eluted with physalaemin several minutes earlier than uperolein (Fig. 3b). These data suggest that even though PSLI seemed to be larger than physalaemin, its ionic properties are very similar, if not identical, to those of physalaemin.

PSLI differs from substance P on the basis of several criteria: specificity of antiserum PS-1 (Table 1); tissue distribution and cellular localization²¹; substance P has not been localized in Brunner's glands²⁷ which contain cells immunopositive to the COOH terminus of gastrin²⁸ and bombesin²³; and PSLI co-eluted only with physalaemin on HPLC (Fig. 3b).

The physiological effects of physalaemin may indicate a possible role for PSLI. Physalaemin affects mucous production *in vitro*²⁹, and *in situ* stimulates secretion of the lachrymal and salivary glands^{3,30} with the secretion of K⁺ and reabsorption of Na⁺ (ref. 31). These activities may account for the presence of PSLI in tissues with mucous production, Brunner's glands and in mucosal layer of trachea and oesophagus. Physalaemin produces acute hypotension by vasodilation^{2,3,6}, and a spasmogenic action on extracellular smooth muscle^{2,3} which suggests that locally released PSLI could regulate muscle tone; for example, in gastro-oesophageal sphincter⁸ or enteric plexi²² the relaxation induced by VIP may be antagonized by PSLI. The presence of PSLI nerves in the vicinity of acinar cells suggests that it could interact on specific physalaemin membrane receptors on acinar cells³² leading to cellular secretion³²⁻³⁴. Thus the release of an endogenous PSLI may be involved in different physiological functions depending on its neuronal or cellular location.

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Dual ionic controls for the activation of protein synthesis at fertilization

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The general metabolic activation of the sea urchin egg at fertilization is dependent on a release of intracellular stores of calcium and the subsequent transient elevation of intracellular Ca²⁺ (refs 1-3). However, this elevation does not by itself lead to increased macromolecular synthesis and development but initiates steps which result in a long-term elevation of intracellular pH (refs 4-6). Among the developmental processes dependent on the elevation of intracellular pH is the large acceleration in the rate of protein synthesis at fertilization⁶. Weak penetrating bases such as ammonia can be used to mimic the processes resulting in an increase in intracellular pH and so show the corresponding increases in protein synthesis rate⁶⁻⁹. Conversely, it is possible to demonstrate a gradual but complete shut down of protein synthesis if the intracellular pH is reduced to the unfertilized level with penetrating weak acids⁶. However, the rate of protein synthesis in ammonia-activated eggs lags behind that of fertilized controls even though ammonia activation can result in an intracellular pH increase greater than occurs in the fertilized egg^{6,8,10}. This result has led to the suggestion that factors other than intracellular pH may be regulating protein synthesis following fertilization^{6,10}. To investigate the possibility that the Ca²⁺ transient may have such a role, we measured the rate of amino acid incorporation in eggs that were activated in various ionic conditions which enabled the effects of Ca²⁺ and pH changes to be studied separately. Our results, reported here, show that if intracellular pH is elevated, increases in intracellular Ca²⁺ play an additional part in the activation of protein synthesis at fertilization.

Figure 1 shows the amount of ³H-valine incorporated after activation in these different ionic conditions. Eggs were preloaded with ³H-valine to circumvent problems of differential uptake of label in different experimental conditions. Fertilization typically results in a 10-30-fold stimulation of protein synthesis over the unfertilized rate. The variation between experiments stems from differences in both the basal unfertilized rate and the degree of stimulation resulting from activation.

Fertilization results in an increase of intracellular pH of about 0.45 pH units⁵. This seems to occur by a mechanism by which protons are released from the egg in the presence of external Na⁺ (ref. 4). To determine how Ca²⁺ release in the absence of an

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intracellular pH increase would affect the protein synthesis rate, eggs were activated with the Ca^{2+} ionophore A23187 in zero-sodium (0-Na^+) artificial seawater. The ionophore causes the release of intracellular Ca^{2+} and the discharge of the cortical granules, but in the absence of external Na^+ there is no intracellular pH increase¹¹, and the rate of protein synthesis is the same as in the unfertilized controls (Fig. 1a). Similarly, if eggs are fertilized in seawater and immediately transferred to 0-Na^+ artificial seawater before the onset of the Na^+ -dependent H^+ efflux, there will be a Ca^{2+} release without an increase in intracellular pH. Protein synthesis remains at the unfertilized rate until Na^+ is added back and the intracellular pH increases (Fig. 2). Thus, a Ca^{2+} release in the absence of a pH rise does not stimulate protein synthesis.

Treatment with 10 mM NH_4Cl increases the intracellular pH by about 0.80 pH units and does so by an entirely different mechanism^{5,9}. NH_4Cl in alkaline solutions will generate ammonia, which passively diffuses across the egg plasma membrane raising the intracellular pH, bypassing the transient

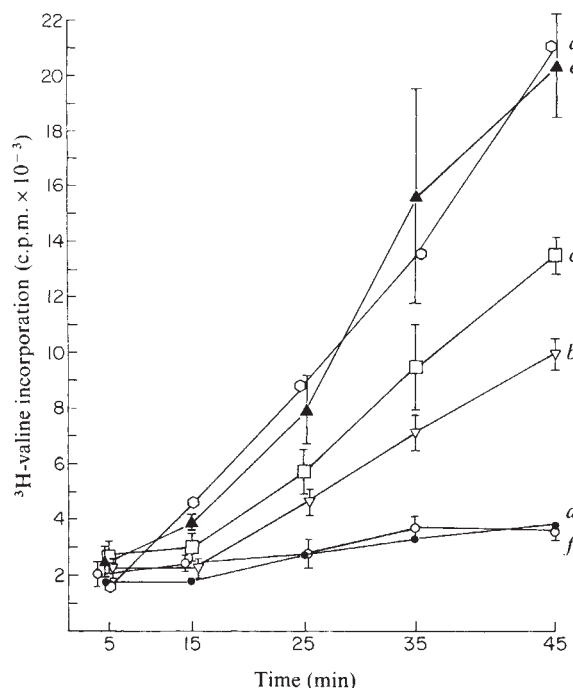


Fig. 1 Total ^3H -valine incorporation over a 45-min period starting 5 min after activation in various ionic media. The mean incorporation of label into unfertilized eggs from four experiments was determined for each time point. The ratio of this mean to the unfertilized rate in each of these four experiments was used to normalize the data presented for the different ionic conditions. The plotted data points represent the mean of the normalized incorporation data. The value shown represents the amount of incorporation $\pm$ s.e.m. for four repeats of this experiment testing five different conditions plus fertilization. The standard errors for fertilization have been omitted and the time points slightly spread out for clarity. *a*, Eggs activated by 2.5 μM A23187 in 0-Na^+ artificial seawater: intracellular Ca^{2+} release without an intracellular pH rise. *b*, Eggs activated by 10 mM NH_4Cl in 0-Ca^{2+} artificial seawater: a rise in intracellular pH without a Ca^{2+} release or influx. *c*, Eggs activated in 10 mM NH_4Cl in 0-Na^+ artificial seawater: an intracellular pH rise with some Ca^{2+} influx. *d*, Eggs activated in 10 mM NH_4Cl and 2.5 μM A23187 in 0-Na^+ artificial seawater: both an intracellular pH rise and an intracellular Ca^{2+} release. *e*, Eggs fertilized normally in natural seawater for comparison purposes. *f*, Unfertilized eggs in natural seawater. Artificial seawater: 484 mM NaCl, 10 mM KCl, 27 mM MgCl_2 , 29 mM MgSO_4 , 11 mM CaCl_2 , 2.4 mM NaHCO_3 , pH 8.0. 0-Na^+ artificial seawater had choline Cl substituted for NaCl and KHCO_3 for NaHCO_3 . 0-Ca^{2+} artificial seawater had 16 mM NaCl substituted for CaCl_2 and 1 mM EGTA added. Eggs of the sea urchin *Lytechinus pictus* were obtained and preloaded as described previously⁶. Briefly, a 0.75-ml volume of packed dejellied eggs was preloaded in 15 ml of seawater containing 5 $\mu\text{Ci ml}^{-1}$ [2, 3- ^3H]valine (Schwarz-Mann, 26 Ci mmol^{-1}) for 45 min. The eggs were then washed twice in seawater and once in the test solution before resuspending to a 0.1% concentration in the test solution. Aliquots (2 ml) were taken at 10-min intervals and processed for scintillation counting as described previously⁶.

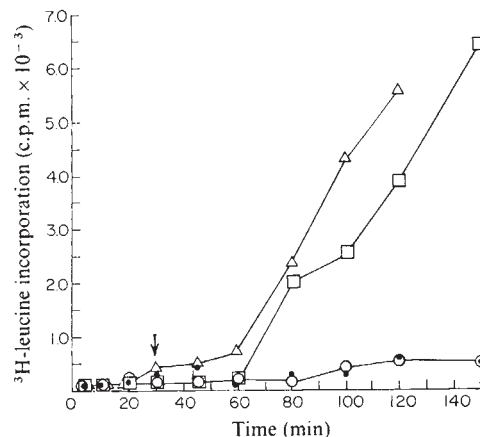


Fig. 2 Total ^3H -leucine incorporation over a 150-min period showing the effect of Na^+ on the acceleration of protein synthesis at fertilization. ●, Unfertilized eggs in seawater; ○, Eggs fertilized in normal seawater and completely transferred to 0-Na^+ seawater 50 s after fertilization; △, fertilized eggs in seawater; □, eggs fertilized in normal seawater and completely transferred to 0-Na^+ seawater 50 s after fertilization. At the arrow, 30 min after fertilization, eggs were transferred back to normal seawater. Eggs of the sea urchin *Strongylocentrotus purpuratus* were treated with 5 mM dithiothreitol (DTT), pH 8.5, until test aliquots showed that fertilization would not produce a fertilization envelope. Eggs were then preloaded with 5 $\mu\text{Ci ml}^{-1}$ [4, 5- ^3H]leucine for 1 h. Aliquots (2 ml) of a 1% suspension of eggs were put into polylysine-coated Petri dishes, one for each time point. This treatment firmly attaches the eggs to the Petri dish where they will remain even if fertilized because the DTT treatment prevents the elevation of the fertilization envelope. These attached eggs could then be quickly and completely transferred in and out of the 0-Na^+ seawater. The attachment of DTT-treated eggs to polylysine-coated surfaces delays the response of protein synthesis to fertilization by 20 min in both the experimental and the control conditions. At each time point the seawater was removed and 5 ml of 10% trichloroacetic acid added to the Petri dish. At the completion of the experiment the eggs were scraped from the dishes using rubber policeman, collected on glass fibre filters and processed as described previously⁶.

intracellular Ca^{2+} release and not causing the discharge of the cortical granules. However, NH_4Cl in seawater containing Ca^{2+} does provoke a transient Ca^{2+} influx¹². NH_4Cl activation in seawater or zero 0-Na^+ artificial seawater (both of which have the normal amount of Ca^{2+}) will result in similar stimulations of protein synthesis to levels less than those achieved by fertilization. If extracellular Ca^{2+} is present during NH_4Cl activation there will be a measurable influx of Ca^{2+} , which although insufficient to cause discharge of the cortical granules, does affect the level of protein synthesis. If protein synthesis is compared in eggs activated in 0-Na^+ seawater where Ca^{2+} influx can occur (Fig. 1c) and in 0-Ca^{2+} artificial seawater where there is no measurable Ca^{2+} influx, there is a significantly lower level of protein synthesis in the absence of the Ca^{2+} influx (Fig. 1b).

If eggs are treated with A23187 and 10 mM NH_4Cl in 0-Na^+ artificial seawater, there will be both an intracellular Ca^{2+} release caused by the ionophore and an intracellular pH increase caused by the NH_4Cl . The rate of protein synthesis approaches that of normal fertilized eggs (Fig. 1d). Thus, Ca^{2+} release and an increase in intracellular pH, although induced by mechanisms other than the normal ones used by the egg at fertilization, will lead to the fertilized level of accelerated protein synthesis. The higher levels of amino acid incorporation with both a Ca^{2+} and pH stimulation are unlikely to be due to a change in amino acid pools because pulse labelling experiments give an identical ordering of incorporation rates (unpublished results).

Thus, Ca^{2+} release in the absence of an intracellular pH increase does not stimulate protein synthesis. A pH increase in the absence of a Ca^{2+} release will yield a partial stimulation of protein synthesis. A small Ca^{2+} influx in the presence of a pH increase will yield higher levels of protein synthesis. Only in the presence of both an intracellular Ca^{2+} release and a pH increase will protein synthesis be maximally stimulated.

Brandis and Raff¹⁰ have provided evidence for an increase in the peptide elongation rate associated with fertilization that is not seen with NH_4Cl stimulations. It is possible that the

increased elongation rate is dependent on the Ca^{2+} release at fertilization. However, it is difficult to be sure of such an interpretation of the differences between NH_4Cl activation and fertilization because (1) the rate of protein synthesis is changing very rapidly, (2) it is affected by the cell cycle changes, and (3) extracellular Ca^{2+} enters during NH_4Cl activation in seawater. In our experiments, however, the magnitude of the response to NH_4Cl stimulation with varying amounts of Ca^{2+} entry or release clearly show that if the intracellular pH is elevated, Ca^{2+} plays an additional role in accelerating the rate of protein synthesis at fertilization.

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Mutagenic deamination of cytosine residues in DNA

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Spontaneous deamination converts cytosine to uracil, which is excised from DNA by the enzyme uracil-DNA glycosylase, leading to error-free repair. 5-Methylcytosine residues are deaminated to thymine, which cannot be excised and repaired by this system. As a result, 5-methylcytosine residues are hotspots for spontaneous transitions, as demonstrated in the *lacI* gene of *Escherichia coli*. We show here that in bacteria which lack uracil-DNA glycosylase (Ung^-) and cannot excise uracil residues from DNA, the rate of spontaneous transition at cytosine residues is raised to the hotspot rate at 5-methylcytosine residues. These studies provide direct evidence that the deamination of cytosine is a significant source of spontaneous mutations.

The observation by Lindahl that potentially mutagenic cytosine deamination occurs at observable rates in *in vitro* physiological conditions led to the discovery of uracil-DNA glycosylase¹. This enzyme initiates the repair of uracil from DNA by a 'base-excision' repair pathway^{2,3}. *E. coli* uracil-DNA-glycosylase-deficient mutant strains⁴ have been shown to have a weak G:C→A:T specific mutator phenotype⁵. Presumably, a G:U mismatched base pair resulting from cytosine deamination generates an A:U base pair after replication. Thus, one of the physiological roles of uracil-DNA glycosylase may be to repair such pre-mutational lesions resulting from deamination.

A more general description of the possible significance of cytosine deamination as an important mutagenic process comes from studies of the *E. coli lacI* gene⁶⁻⁸. Large numbers of independent *lacI* mutations have been characterized by genetic and biochemical methods. Of spontaneous base substitutions leading to nonsense mutations, approximately 75% arise by

Table 1 Frequency of spontaneous I^- mutants ($\times 10^6$)

Strain	Approx. I^- mutant frequency	% Amber mutants
GM1	2.5	1.0
BD1322	2.5	1.3
BD1323 (Ung^-)	2.5	4.6

I^- mutants were selected and screened against different suppressors to detect amber mutations, as described previously^{7,8}. BD1322 is an Ung^+ derivative of the Ung^- strain BD1323. It is isogenic to GM1.

Table 2 Distribution of amber mutations in Ung^+ and Ung^- strains

		No. of occurrences		
Base substitution	Site	Ung^+ GM1	Ung^- BD1323	Ung^- BD1323 (2AP)
G:C→A:T	A5	0	2	0
	A6*	51	4	39
	A9	8	18	4
	A15*	37	0	29
	A16	9	1	2
	A19	11	2	1
	A21	8	2	0
	A23	8	7	2
	A24	10	1	5
	A26	6	2	2
	A31	1	2	3
	A33	5	4	1
	A34*	12	3	11
	A35	1	2	0
Total G:C→A:T mutations		167	50	99
Transversions		55	4	0
Total amber mutations detected		222	54	99

Distribution of amber mutations in Ung^+ and Ung^- strains, showing the distribution found in GM1 and in BD1323 with and without 2-aminopurine (2AP). The number of occurrences refers to the number of mutations detected at a given locus in the collections of 222, 55 and 99 *lacI* mutations, respectively. The characterization of the GM1 amber mutations has been reported previously⁶⁻⁸. The I^- collection from BD1323 was analysed in the same manner.

* Presence of 5-methylcytosine at the mutational site⁶.

G:C→A:T transitions⁶⁻⁸. Two highly mutable 'hotspot' sites are conspicuous among 14 G:C→A:T amber mutation sites in the *lacI* gene; half of the observed amber transition mutations occur at these two sites. DNA sequence studies have demonstrated the presence of 5-methylcytosine residues at the hotspot sites⁶. It is clear that cytosine methylation increases the mutagenic susceptibility of a cytosine residue, presumably because of the inability of the cell to repair the deamination product, thymine.

We have studied the effect of the *ung* mutation, which results in the loss of uracil-DNA glycosylase activity, on the distribution of I^- nonsense mutations. The *ung-1* allele was inserted by P1 transduction into the *E. coli* GM1 used in previous studies of the *I* gene⁶⁻⁸. Table 1 shows that the frequency of amber mutations increased almost fourfold in the presence of the *ung*⁻ mutation. Thus, a deficiency of uracil-DNA glycosylase led to an increase in the frequency of mutations due to base substitutions. The I^- amber mutations were assigned to one of the known amber sites by deletion mapping and by examining the response to different amber suppressors, as described previously^{7,8}.

G:C→A:T transitions generate amber mutations at 14 different sites in the *lacI* gene. This represents 38% of the 37 characterized amber mutation sites in *lacI* which can be derived by a single base change. As Table 2 shows, 93% of the amber mutations detected in the Ung^- background arose by way of a G:C→A:T transition. In Ung^+ cells (GM1), 75% of all the amber mutations were G:T→A:T transitions, principally due to the 5-methylcytosine hotspots A6 and A15 (and to a lesser extent A34).

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A comparison of the distribution of the $G:C \rightarrow A:T$ mutations yields a more striking difference between ung^+ and ung^- cells. The general increase of transition mutations in the ung^- cells results from an increase in transitions at non-methylated cytosines, bringing them up to the level of the 5-methylcytosine sites. Thus, the 5-methylcytosine sites (A6, A15 and A34) no longer appear as hotspots. Because uracil-DNA glycosylase has been shown to function in the base excision repair of uracil, these results demonstrate that cytosine deamination occurs *in vivo*; the failure to repair these lesions leads to an increase in the mutation rate.

Methylcytosine residues have also been shown to be hotspot loci when cells are mutagenized with 2-aminopurine (2AP)⁶⁻⁸. Could 2AP be stimulating the deamination of cytosine and methylcytosine residues? Perhaps the 2AP incorporation into DNA perturbs the structure of the DNA, making it more susceptible to cytosine deamination. Lindahl has shown that the DNA-cytosine deamination rate constant increases more than 100-fold when DNA is denatured⁹. This model does not necessarily assume the actual mispairing of 2AP with cytosine; formation of a 2AP:T base pair might also induce the deamination of nearest-neighbour cytosine residues. We have tested this hypothesis. The right-hand column of Table 2 shows that the ung mutation does not affect the mutability of methylcytosine residues compared with other cytosine residues in the presence of 2AP. Thus, with the Ung^- strain, 80% of the 2AP-induced amber mutations are at the three methylated cytosines, which compares favourably with the wild-type Ung^+ (GM1) strain, in which 60% of the transitions resulting in amber mutations are at the methylated site, and with GM1 + 2AP⁶⁻⁸, in which 85% of the amber mutations are at the methylated cytosines. These results argue that 2AP does not induce deamination.

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Visualization of an unwound DNA duplex

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Certain dyes^{1,2} and drugs^{3,4} with planar aromatic components can intercalate these into stacks of base pairs and thereby bind tightly to DNA duplexes. Intercalation at one site usually precludes intercalation between the base pairs immediately adjacent⁵. This exclusion implies that two distinct nucleoside conformations are needed in the dinucleoside phosphates which include the intercalation site. The simplest distinction would involve no more than quantitative differences in the (usually *anti*) conformations at the glycosidic bonds⁶. This could be reinforced by additional, qualitative differences in the furanose ring puckerings (C-2'-*endo* and C-3'-*endo*)⁷. For the most

pronounced difference there could be qualitative differences (*syn* and *anti*) in the conformations of the glycosidic bonds as well as in the conformations of the sugar rings. The model discussed here is an example of this most emphatic distinctiveness, as the nucleosides at the 5' ends of the intercalation sites are C-3'-*endo* and *syn* and at the 3' ends are C-2'-*endo* and *anti*. X-ray diffraction analysis suggests that a completely unwound allomorph of the DNA duplex can persist in oriented fibres when stabilized by certain platinum-containing intercalators. In the untwisting of (usually) right-handed DNA double helices, unwound duplexes are presumably fleeting intermediates.

Previous precise visualizations of intercalation in DNA came from extrapolatory modelling⁶⁻⁹ inspired mainly^{8,9}, but not exclusively^{6,7}, by X-ray diffraction studies of crystals containing self-complementary dinucleoside monophosphate duplexes complexed with intercalators. The accuracy of particular models of intercalation into DNA itself can be determined^{10,11} by X-ray diffraction studies of fibres of calf thymus DNA saturated with a variety of platinum derivatives. The fibres are uniaxially oriented and their diffraction patterns, of which Fig. 1 is an example, show a layer line with 10.2 Å spacing, in striking agreement, as the original authors^{10,11} emphasized, with a neighbour exclusion model. This is exactly the length expected of a unit of structure involving an intercalator sandwiched between two base pairs (Fig. 2a) which are then directly stacked on the base pairs of the next intercalator sandwich (Fig. 2b-d). Also, some of the fibres are polycrystalline so that the layer lines on the diffraction patterns are populated with fairly sharp Bragg reflections. Their spacings and intensities are satisfactorily explained by a model whose molecular conformation and packing are rather different from those proposed earlier¹⁰. An important conclusion of our study is that the DNA duplexes in these complexes are completely unwound and therefore not double helices. We have therefore an unprecedented opportunity to visualize a most unusual allomorph of double-stranded DNA.

We have confined our detailed analysis to the diffraction pattern (Fig. 1) provided by the DNA · [(bipy)Pt(en)]²⁺ complex; however, our interpretation of the patterns from the DNA complexes with the other platinum-containing intercalators would be essentially the same.

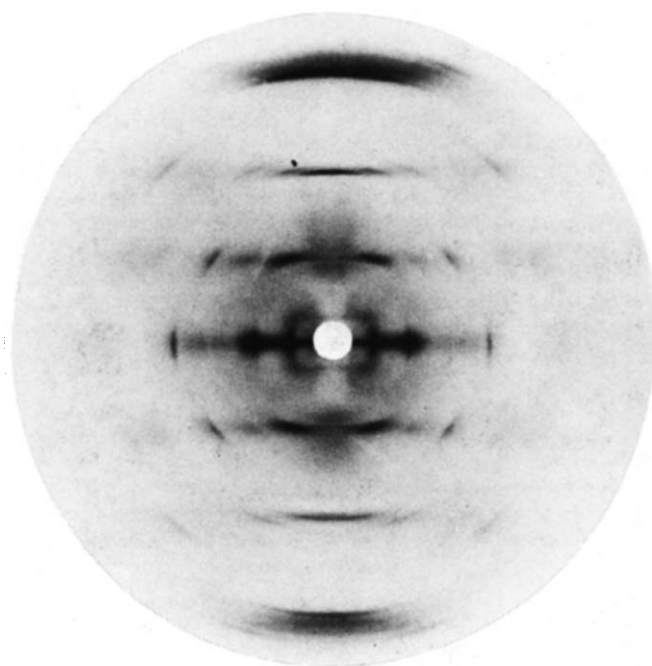


Fig. 1 The X-ray diffraction pattern from a uniaxially oriented, polycrystalline fibre of calf thymus DNA saturated with intercalating [(bipy)Pt(en)]²⁺ cations. The layer line spacings indicate an axial repeat of 10.2 Å. The Bragg reflections can be indexed on the basis of a rectangular unit cell 11.5 × 20.0 × 10.2 Å³.

Table 1 Comparison of the conformation angles of L DNA in the DNA · [(bipy)Pt(en)]²⁺ complex with those of S DNA

Conformation angle (deg)	L DNA		S DNA*	
	XpX†	XpX	CpG	GpC
$\theta(\text{C4}'-\text{C3}'-\text{O3}'-\text{P})$	-84	171	-72	-103
$\theta(\text{C3}'-\text{O3}'-\text{P}-\text{O5}')$	98	125	102	-91
$\theta(\text{O3}'-\text{P}-\text{O5}'-\text{C5}')$	82	-60	52	-110
$\theta(\text{P}-\text{O5}'-\text{C5}'-\text{C4}')$	-162	-133	-153	-168
$\theta(\text{O5}'-\text{C5}'-\text{C4}'-\text{C3}')$	180	-139	178	54
$\theta(\text{C5}'-\text{C4}'-\text{C3}'-\text{O3}')$	76	147	76	147
$\theta(\text{O4}'-\text{C1}'-\text{N9}-\text{C4})^\dagger$	26	167	89	—
$\theta(\text{O4}'-\text{C1}'-\text{N1}-\text{C2})^\dagger$	26	167	—	-159

L DNA is the trivial name we have given to the ladder-like structure, and S DNA is the polymeric version of the left-handed duplex.

* From ref. 14.

† X can be a purine or a pyrimidine.

The 30 independent Bragg reflections in Fig. 1 can be indexed adequately on the basis of a rectangular unit cell with dimensions (s.d.) $a = 19.95(15)$ Å, $b = 11.52(05)$ Å, $c = 10.20(05)$ Å. There are no reflections requiring the (six times) larger trigonal unit cell suggested earlier¹⁰.

Clearly, the new unit cell cannot accommodate quasi-cylindrical DNA double helices with diameters at all like the traditional values of 21 ± 4 Å. This should not be surprising because

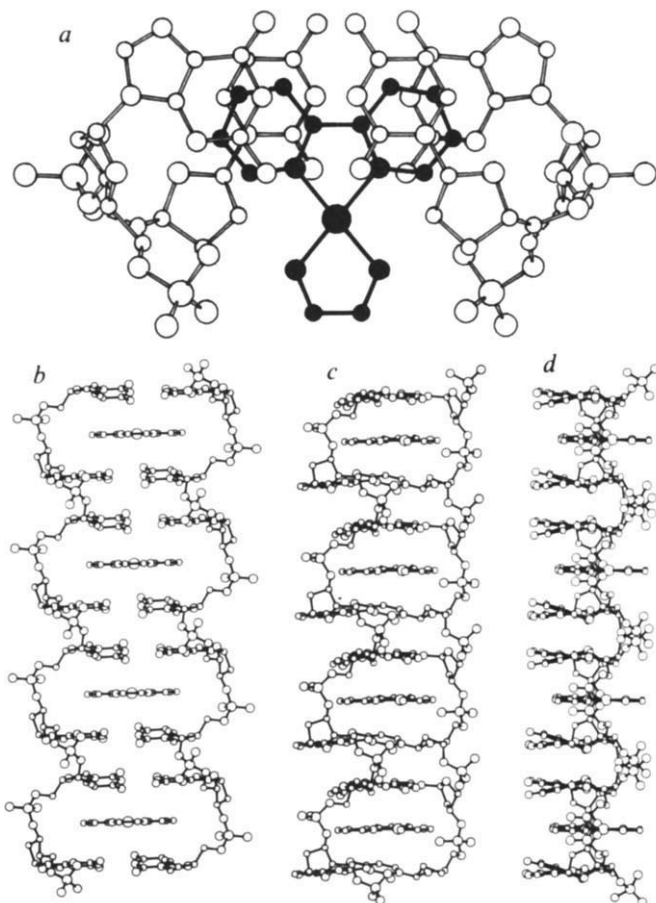


Fig. 2 The unwound DNA duplex with its stabilizing intercalators. *a* Shows the view parallel to the *c* axis of the stacking arrangement of an intercalator between two base pairs. Because this unit is repeated by simple translation along the *c* axis and the base pairs are almost perpendicular to this axis, the base pairs in the excluded site overlap one another in the same way as appears here; *b-d* contain three views perpendicular to the *c* axis, including the view down the diad axis (*b*) and views after successive rotations of 45° about *c*.

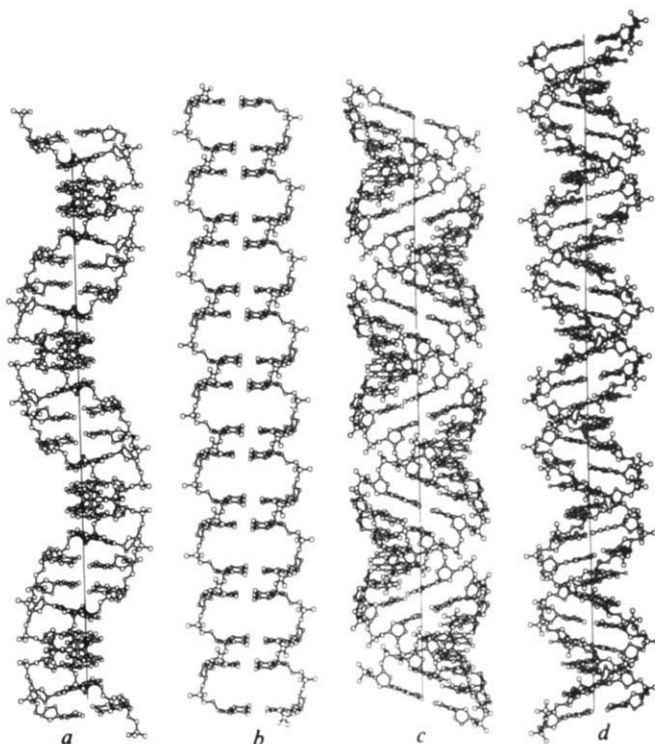


Fig. 3 Comparison of the morphologies of the most underwound (left-handed) DNA double helix (*a*), the unwound duplex (*b*), a right-handed double helix of the A family (*c*) and the most overwound (right-handed) double helix (which belongs to the B family) (*d*).

the layer line spacings also are not compatible with a helical molecular structure. If successive, 10.2 Å-long units of structure were related by helical symmetry there should be layer lines with spacings $10.2(N/n)$ Å where N and n are integers and $N > 1$. In fact, $N = 1$ and all the layer lines have spacings $(10.2/n)$ Å, $n = 1, 2, 3, \dots$, implying that the structural unit is repeated by simple translation along the chain axis.

Bond *et al.*¹⁰ were unable to build a stereochemically reasonable model duplex containing intercalated dinucleotide structural units except as N -fold helices with $N \geq 7$. In these experiments all glycosidic conformations were *anti*. By contrast, we have found that it is quite easy to construct a stereochemically acceptable model of the required '1-fold helix' if the glycosidic conformations along the polynucleotide chain are alternately *syn* and *anti*. The best arrangement associates a C-3'-*endo* furanose with the *syn* nucleoside and a C-2'-*endo* furanose with the *anti* nucleoside as in the left-handed screw structures observed with certain oligonucleotides^{12,13} and DNA polymers¹⁴. Indeed, the segment involving stacked base pairs has the same kind of nucleotide conformations (see Table 1) as CpG in the left-handed (6_5) helical form of poly(dG-dC) · poly(dG-dC) (ref. 14). Not surprisingly, the GpC conformation in the 6_5 helix is not retained in the nucleotide which has to straddle the intercalation site. Extension of the chain and the achievement of no net winding after two nucleotides is contrived (see Table 1) by having *trans* conformations around C3'-O3' (*gauche minus* in the 6_5 helix), P-O3' (*gauche minus* in the 6_5 helix) and C4'-C5' (*gauche plus* in the 6_5 helix).

In constructing the model of the DNA duplex in this complex, we assumed that the chains are antiparallel and conformationally identical. There are, therefore, diad axes half way between successive base pairs and perpendicular to the long axis of the molecule. We have further assumed that the diad axis within the [(bipy)Pt(en)]²⁺ cation coincides with the DNA diad which is in the intercalation site. This leaves two possible dispositions for the intercalator. We find that the one which places the platinum atoms in the minor groove (Fig. 2*a*) seems to be preferable from both steric and diffraction considerations.

The small cross-section of the unit cell clearly shows that it can be traversed by no more than one intercalated duplex, oriented so that the minor groove is approximately bisected by the *b* axis. The diffraction pattern has some continuous intensity as well as Bragg reflections on each layer line, suggesting a 'statistical' crystal structure¹⁵. The best arrangement we have found has rows of identically oriented molecules lined up along *a* 20-Å apart. About one-third of the rows separated by 11.5 Å in the *b* direction will also be mutually translated $c/2 = 5.1$ Å in the *c* direction and the molecules in the two rows mutually rotated by 30° about *c*. Such an arrangement would result in no steric anomalies and provide a good explanation of both the Bragg and the non-Bragg diffraction. For the Bragg reflections the conventional crystallographers' residual *R* has an encouragingly low value of 0.31 after a least-squares refinement¹⁶ of the complex in which the polynucleotide chains are treated as a linked-atom system and the [(bipy)Pt(en)]²⁺ intercalator as a rigid body free to move along and rotate about the diad axis which it is assumed to share with the DNA duplex.

In the refined structure the mean plane of each base pair of the DNA duplex is tilted 4° from being perpendicular to *c*. The bases in the base pairs are not coplanar but are 'propeller-twisted' with an angle of 22° between their planes. The intercalators are also tilted (in their case 5°).

Polynucleotide duplexes with Watson-Crick base pairs have quite polymorphic secondary structures (Fig. 3). Apparently this is not generally accepted because few of the many and varied allomorphs have been exploited in visualizing DNA and RNA duplexes in dynamic and interactive modes. Yet it is almost 30 yr since the identification¹⁷ of A and B DNA indicated what a wide range of structure might be achieved by polynucleotide duplexes. A and B-DNA are helical structures with very distinct axial translations (*h*) and rotations (*t*) per nucleotide: *h*, *t* = 2.56 Å, 32.7° in the former and 3.38 Å, 36.0° in the latter. Many allomorphs have now been trapped in oriented fibres and had their conformations defined¹⁸. All were right-handed double helices with $2.56 \text{ Å} \leq h \leq 3.38 \text{ Å}$ and $30.0^\circ \leq t \leq 45.0^\circ$. Recently, these ranges have been extended dramatically by the discovery of further wound right-handed helices¹⁹ (with *t* = 48.0°) and the underwound (left-handed) helices¹⁴ (with *t* = 30.0°) anticipated by the NMR²⁰ and single crystal^{12,13} studies of oligo(dG-dC) · oligo(dG-dC).

The present structure with $\langle h \rangle = 5.1$ Å and $\langle t \rangle = 0.0^\circ$ is a further example of the polymorphism of polynucleotide duplexes. Clearly, the partially stacked polynucleotide component of the complex could have only a brief lifetime in the absence of the stabilizing intercalator. Therefore, we have visualized one of the higher energy states available to DNA. In the present context this state has been exploited by a synthetic intercalator, but obviously it is a state which is available also for interaction with native molecules.

The structure of this complex also adds pointedly to the contention^{6,8,9} that there is no single conformational strategy whereby polynucleotide duplexes contrive to accommodate intercalators. Further, the local unwinding resulting from intercalation can be much larger than is often supposed.

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Metallothionein occurs in roots of *Agrostis* tolerant to excess copper

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Metallothioneins have an important function in metal metabolism in animals¹. These low molecular weight proteins have high contents of half-cystinyl residues (up to 33%) which can bind metals such as Zn, Cu, Cd, Hg or Ag in mercaptide complexes¹. In normal conditions animal tissues have low concentrations of metallothioneins, but these increase on administration of metal²⁻⁴, a reaction which is consistent with their suggested role in detoxification mechanisms^{2,5,6}. Various plant genotypes exhibit tolerance to excesses of metals⁷, and Jowett⁸ proposed that such genotypes contain compounds capable of specific metal chelation. Metallothioneins could serve such a role. We report here that copper-thionein occurs in the roots of a clone of *Agrostis gigantea* Roth tolerant to excess copper⁹. This is the first unequivocal demonstration of a metallothionein in a vascular plant. These unusual proteins may have a role in metal metabolism in plants and could be important in the elucidation of the mechanism of metal tolerance.

Apical tillers of clone 4 of *Agrostis gigantea* were transplanted into tanks containing complete nutrient solution and allowed to develop roots for 3 weeks⁹. Plants were then exposed for 1 week to 16 μM CuSO₄ in the nutrient solution. Roots were harvested, washed three times in large volumes of cold deionized water and blotted with paper towelling. The roots were either used at once or frozen in liquid nitrogen for later use. All operations for extracting and isolating proteins were carried out at 0-4 °C with solutions purged with N₂ and in enclosures flushed with N₂. A typical separation of heat-denatured root extract on DEAE Biogel A is shown in Fig. 1. The protein was resolved into material not adsorbed to the ion exchanger (fraction A) and a prominent peak of copper-binding protein that was eluted only after adding NaCl (fraction B). Metallothioneins characteristically adsorb strongly to DEAE^{3,10}. Fraction B from this and other isolations was purified by electrophoresis on 3-mm thick gel slabs of 10% w/v polyacrylamide with Tris-glycine pH 8.3 as electrode buffer¹¹. In a typical run where 165 mg fraction B product was applied, a single dark brown band 5 mm wide was evident after migrating 10 cm. The dark brown material was eluted from the gel and analysed for molecular weight (MW), amino acid composition and metal content. Fraction B protein showed a major band at a MW of 14,400 and lesser bands at 9,800, 6,200 and 5,400 after SDS-polyacrylamide gel electrophoresis.

From the amino acid composition listed in Table 1 it is clear that fraction B protein has a high half-cystine content, a key feature of metallothioneins^{1,2,10}. Acidic amino acids predominated and, along with cysteine, glycine and serine, accounted for 67.5% of the residues compared to 70.2% for yeast Cu-thionein¹². The ratio of half-cystine to copper was 1.9-2.4 to 1 in accord with the stoichiometry found for Cu-thionein from

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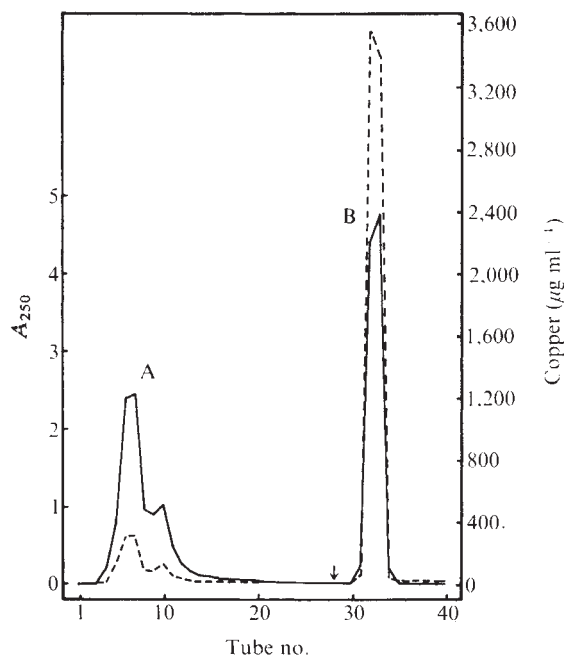


Fig. 1 Ion-exchange chromatography of copper-binding proteins from *Agrostis gigantea*. 60 g of frozen roots were homogenized with 60 ml 10 mM Tris-HCl, pH 8.0, 5 mM NaCl, 5 mM 2-mercaptoethanol in a mortar and pestle. The homogenate was strained through cheesecloth and centrifuged at 20,000g for 30 min. Heat denaturation of the supernatant was done by rapid heating at 60 °C for 3 min while stirring under a stream of N₂. The preparation was cooled in an ice bath and centrifuged at 20,000g for 30 min. The supernatant was concentrated to 5 ml by ultrafiltration on a Diaflo UM2 membrane (Amicon) and washed three times with a total of 50 ml 5 mM Tris-HCl, pH 8.6. The washed concentrate was applied to a DEAE Biogel A column (1.5 × 25 cm) equilibrated with 5 mM Tris-HCl, pH 8.6. Proteins were eluted with the same buffer at a flow rate of 70 ml h⁻¹ with fraction volumes of 8 ml. At tube 28 (arrow) the elution buffer was changed to one containing 1 M NaCl. —, Protein; -----, copper.

Table 1 Amino acid composition of copper-binding proteins from *Agrostis gigantea*

	Fraction A	Fraction B
Half-cystine*	4.8	18.4
Aspartate	12.8	10.0
Glutamate	13.4	19.0
Glycine	11.7	11.9
Serine	8.6	8.2
Threonine	5.9	2.9
Proline	7.5	5.8
Alanine	9.0	6.0
Valine	6.4	4.2
Methionine	1.0	0.7
Isoleucine	3.6	1.5
Leucine	5.9	3.6
Tyrosine	1.1	0.6
Phenylalanine	2.0	1.5
Lysine	3.5	3.3
Histidine	1.4	0.8
Arginine	1.4	1.6

The material in fractions A and B from DEAE Biogel A separations was purified electrophoretically on slab gels, dialysed exhaustively against 5 mM Tris-HCl, pH 8.6, and then hydrolysed in N₂-saturated 6 M HCl at 110 °C for 24 h. Values are expressed as per cent of the total number of residues.

* Only 0.6–0.7 residue % was found as cysteic acid.

thioneins¹⁴. This divergence raises the possibility that the proteins binding copper might be set apart from the Cd- and Zn-thioneins even though they all have the unique function of binding abundant metal.

The nature of the Cu-binding material in fraction A is less clear. The amino acid composition of electrophoretically purified fraction A protein (Table 1) indicates that it is not an aggregate of Cu-thionein as has been suggested by others^{13,15,16}. The copper content was much lower at a cysteine to copper ratio of 0.5:1, yet zinc comprised only 3–6% of the bound metal. Electrophoresis in SDS gels showed bands of 5,400, 11,600 and 52,300 MW against a background of Coomassie blue-staining material which reached the start of the gel.

Quantitative estimates of the amount of Cu-thionein in the roots of *Agrostis gigantea* were not possible. Protein estimations by either biuret or dye binding¹⁷ methods were increasingly unreliable as the metallothionein was purified. Occlusion of material on the ultrafilter gave copper losses of 12–17% and there were even greater losses during dialysis of protein concentrates. More reliable methods are required to determine how much Cu-thionein occurs in the roots of *Agrostis gigantea* before any more can be inferred about its involvement in the mechanism of copper tolerance.

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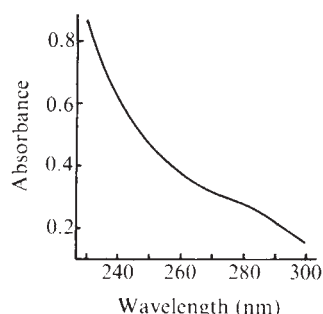


Fig. 2 UV absorption spectrum of fraction B protein in 5 mM Tris-HCl, pH 8.6.

MATTERS ARISING

Superheavy-element fission tracks in iron meteorites

BULL'S careful search¹ for superheavy element (SHE) fission tracks in silicate inclusions within iron meteorites seems to have given a null result. He finds an upper limit of 10^{-12} kg per kg for the original abundance of SHEs in the class 1A Odessa iron meteorite. As this is many orders of magnitude below what we postulated² from considerations of early heat sources in the Moon and from a highly speculative interpretation of elemental abundances in iron meteorites³, Bull's result may be accepted as proof that superheavy elements either did not exist or did not play any part in the early Solar System. His observations therefore merit further study. His result refers to the date at which track retention becomes possible, that is when the temperature has fallen to $\sim 200^\circ\text{C}$. Now although the Rb–Sr, K–Ar dates of silicate inclusions in iron meteorites range up to $\sim 4,600$ Myr, they are not to be interpreted simply as the 'age' of iron meteorites nor are they the dates of the emplacement of the silicate grains. The process by which silicate inclusions get into iron meteorites is unknown: but they must certainly enter while the temperature of the iron is near to its melting point. Cooling rates of iron meteorites are determined by metallographic study and for the Odessa meteorite it is $1.5^\circ\text{C Myr}^{-1}$ in the range $700\text{--}300^\circ\text{C}$. The meteorite might, therefore, only reach track retention temperatures 800 Myr after the emplacement of the silicate crystals. Taking the half life of SHEs determined from the decay of the ancient lunar magnetic field⁴ as 100 Myr, the original abundance of SHEs in the iron would have decayed by nearly 3 orders of magnitude. This, of course, assumes that the rate of cooling at higher temperatures and at lower temperatures is the same. In fact if, as we suppose, SHEs have played a dominant part in the early thermal history of the parent bodies of meteorites, the cooling rates at higher temperatures may have been slower and the diminution in the abundance of SHEs at the time when track retention became possible greater than we have estimated here. Another possible explanation of Bull's results is, of course, that SHEs on the condensation of grains in the early Solar System were never fully taken up by the iron. This explanation seems probable in the case of the Odessa meteorite as IA iron meteorites have been interpreted as not originating in the iron core in a sizeable asteroid-like body. Probably only in a completely melted sizeable body would

the molten iron, mixing with other crystals, dissolve the SHEs. The IA meteorites have reasonably been interpreted in terms of a model in which iron condenses from the nebula and forms small bodies within a much larger silicate parent body. If this model is correct, then the existence of ^{244}Pu fission tracks would have a simple explanation. Bull's studies ought to be done on the IIAB iron meteorites which are more reasonably assumed to come from the cores of entirely melted parent bodies, but the difficulty is that there are no silicate grains to record fission tracks. *Note added in proof:* We are, of course, aware that if the tracks inside the diopside crystals are due to the decay of Pu^{244} , synthesized with the other elements in stellar nucleogenesis processes just before the condensation of the primeval Solar System nebula, our first argument, that the presumed SHE tracks have disappeared due to annealing processes, collapses. But we wonder whether this identification, which, as Bull so clearly explains, is arrived at by a process of elimination, should be accepted without question. Extrapolation of the Periodic Table¹ does not show that all the SHE are siderophile and perhaps some of them, with longer half life, went into the mineral phase.

We agree that our suggested thermal history are necessarily speculative—as are all others—but the track retention temperatures of minerals, such as the quoted 400°C for diopside, are based on laboratory annealing experiments.⁵ But this extrapolation to 4,000 Myr assumes that the same excitation process can be assumed as in laboratory annealing; but it is characteristic of solid state physics phenomena that processes with different excitation energies are dominant over different ranges of temperature and time.

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BULL REPLIES—Runcorn *et al.* have proposed two explanations for the lack of superheavy element (SHE) fission tracks which I observed in silicates from the Odessa iron meteorite¹.

First, they point out that a thermal history for Odessa can be constructed in which the meteorite reaches track retention temperatures only after a time corresponding to many half lives for the SHEs (taken to be ~ 100 Myr) has elapsed. This explanation, however, fails to account for the large excess of fission tracks distributed throughout the diopside grains in Odessa. These exceed the number accruing through the decay of ^{238}U in the lifetime of the Solar System by a factor of ~ 100 and the most plausible source of this excess is the decay of ^{244}Pu which, significantly, has a half life which is very close to that assumed for the SHEs. This means that any cooling which was slow enough to allow most of the SHEs to decay would also result in the loss of most of the Pu and the very large excess of fission tracks in the diopside would be difficult to account for unless this mineral was very much enriched in Pu relative to U (an initial ratio of Pu/U of ~ 10 would be needed compared to values of $\sim 0.01\text{--}0.1$, inferred from fission Xe measurements on chondritic whitlockites²). As to the thermal history proposed by Runcorn *et al.*, I would point out that the track retention temperature of diopside is higher than 200°C , probably nearer 400°C and that there is considerable uncertainty as to the metallographic cooling rates.

The second explanation by Runcorn *et al.* is that the IA irons never took up many SHEs. This point may be valid in that the group IA meteorites are not typical irons and there is evidence that they were never melted. The pallasites, however, are believed to be fragments of a core boundary region and pallasitic metal probably represents core material. In Brenham the superheavy content of the metal was less than 10^{-15} kg per kg at the time of track retention but unfortunately we cannot as yet place firm constraints on when track retention occurred.

Finally, note that silicate inclusions are occasionally found in other iron meteorites groups and a study of these should provide further clues to the abundance of SHEs in the early Solar System.

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Grand unification theories and the large numbers hypothesis

BARROW¹ has recently used the large number hypothesis (LNH) in connection with the proton lifetime.

I wonder about the meaning of his result in light of the fact that he has based his computation on a closed Friedman universe, while Dirac has explicitly shown that "A model with a maximum size for the Universe is not permitted"², that is, is disallowed by the LNH.

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CURRENT ideas based on SU(5) gauge theory suggest the violation of baryon conservation. The proton lifetime, τ_p , is predicted to have various values between 10^{30} and 10^{37} yr. Barrow¹ has noticed that the dimensionless ratio of τ_p so predicted, to the Planck time $\tau_{p1} = (Gh/c^5)^{1/2}$, is not very different from the Universe baryon number $N \sim 10^{79}$. Assuming that the two numbers are equal, he can then assign to τ_p the more definite value of

$$(hc/Gm_p^2)^{1/2}H_0^{-1} \sim 10^{30} \text{ yr}$$

where m_p is the proton rest mass, H_0 the present Hubble 'constant', and factors of the order of unity have been omitted.

We consider instead of τ_{p1} a time unit that involves a property of the particle itself. The simplest choice is $\tau_m = h/(m_p c^2)$ (so that for particles which remain massless $\tau_m \rightarrow \infty$). Intuitively, τ_m is, of course, the minimum lifetime of one proton before its inertial mass can be measured as $\geq m_p$. Furthermore, we speculate on the lifetime τ'_p for its decay hypothetically to some unspecified particles of lower masses under gravitational interaction. The plausibility that all particles with rest masses have finite lifetimes has been considered³. All may eventually decay to gravitons on sufficiently long time scales for which charge conservation is violated; photons may not be massless. Maybe changeability is so prevalent in the physical world that all symmetries are ultimately broken spontaneously. Now, on dimensional grounds τ'_p may be expected to be given by a similar expression as τ_p , with the mediating boson mass replaced by the Planck mass $(hc/G)^{1/2}$ at which gravitational unification should occur, and with the coupling constant α_x substituted by another which is similarly of the order of $1/137$. The result is $\tau'_p \sim 10^{50}$ yr.

We then find that τ'_p/τ_m is again of the same order as the baryon number in the Hubble sphere (or in the Universe). If $\tau'_p/\tau_h = fN$, where f is some factor such as $3^2/8\pi$ and numerically of the order of unity, then

$$\tau'_p \sim (hc/Gm_p^2)H_0^{-1}.$$

However, some of the cosmological coincidences, such as the above two, may really be coincidences and 'explicable' by the anthropic principle² or speculatively as 'historical' data³. This can be true although others are derivable (for example, the coincidences involving the Universe photon-baryon ratio: from SU(5) theory⁴). Future experimental verifications such as of the value of τ_p and excepting a decreasing G , therefore do not confirm the large number hypothesis. Some large dimensionless numbers may equal H_0^{-1}/τ_m or its square only in the present epoch. If the Universe is indeed closed, it has extremal scale factors which are unrelated to its age expressed in atomic units. Thus the hypothesis is inconsistent with⁵ a closed Friedmann universe¹.

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BARROW REPLIES—A large numbers hypothesis (LNH) is not incompatible with a closed finite universe. The original statement of the LNH by Dirac¹ in 1938 was: "Any two of the very large dimensionless numbers occurring in Nature are connected by a simple mathematical relation, in which the coefficients are of the order of magnitude unity." To derive some predictions from this hypothesis one must accumulate a group of large dimensionless quantities of similar magnitude and equate them. The issue of whether or not a 'closed' universe is compatible with the hypothesis depends crucially on the type of large number that is chosen. Dirac's original¹ choice included the ratio of $e^2 m_e c^3 \sim 10^{-23}$ s, (the time for light to traverse the classical electron radius) to the presently inferred age of the Universe, $t_0 \sim 10^{17}$ s. This particular large number therefore incorporates an explicit time dependence through the changing value of t_0 . The hypothesis that it be equal to other dimensionless collections of traditional constants with similar magnitude requires that one of the latter also possess an explicit time dependence. Dirac chose to incorporate the time dependence into Newton's gravitation 'constant', G . If the Universe were finite (either 'closed' or

'open' but with finite volume through topological identifications), then the LNH would also seem to require the total number of protons in the Universe ($\sim 10^{80}$) to increase as t^2 in violation of energy conservation. For this reason Dirac¹ required an open (infinite) cosmological model. However, it is only the choice of a 'large number' possessing an explicit time dependence that suggests such a conclusion, not the LNH itself.

If one chooses large numbers in a less anthropocentric fashion then one naturally replaces the time scale t_0 by t_m , the proper time to the expansion maximum in a closed universe. The quantity t_m , unlike t_0 , is observer-independent and a fundamental cosmic time. Quantitatively this new choice leaves the value of the relevant large number virtually unchanged ($t_m m_e c^3 / e^2 \sim 10^{40}$) because t_m is within an order of magnitude of t_0 , but qualitatively its consequences are quite different: No varying 'constants', additional conformal degrees of freedom or unconventional physics become involved and a closed universe is actually necessary for consistency. Formulated in this manner the LNH simply claims that otherwise unrelated groups of constants possessing similar dimensionless magnitudes are actually equal. This is why I used the time t_m and a 'closed' universe in my formulation. It is, of course, equally legitimate to pursue the more speculative and complicated course that follows from choosing to incorporate t_0 into the large numbers. An 'open' universe would then be a necessary and testable prediction.

Tang derives an interesting estimate for the time scale of a possible gravitational decay of the proton, τ'_p . It depends linearly on the dimensionless gravitational coupling Gm_p^2/hc . A more natural candidate for this time scale might be provided by existing theory²—the time for a proton to quantum tunnel inside its own Schwarzschild radius, $R_s(p) \sim Gm_p/c^2$, and then evaporate into a state of zero baryon charge by the Hawking effect³⁻⁵. One might crudely estimate this time scale as $\tau'_p \sim [R_s^2(p)nc]^{-1}$ where $n \sim (h/m_p c)^{-3}$ is roughly the nuclear density. This estimate gives a decay time varying as the square of the gravitational coupling and considerably in excess of the lifetime suggested by grand unified gauge theories:

$$\tau'_p \sim \left(\frac{hc}{Gm_p^2} \right)^2 \left(\frac{h}{m_p c} \right) \sim 10^{48} \text{ yr}$$

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BOOK REVIEWS

More uncertainty for the common man

J.S. Bell

THE aim of this book is to "face squarely the full impact of fundamental quantum theory on our conception of the world [and] not simply to review a notoriously difficult branch of modern physics, but to turn instead to broader issues. What is man? What is the nature of reality? Is the Universe we inhabit a random accident or the outcome of a delicate selection process?". And all this for a reader with "no previous knowledge of science or philosophy". Professor Davies does not lack ambition.

In fact, I doubt that the book will mean much to a scientific ignoramus; it is more for a reader whose mind is not quite blank before words like 'electromagnetic', 'atom', 'galaxy' and so on. But, with just a minimum of basic scientific culture, it will be possible to get from the book some idea of what quantum theory is, of what an enormous break it is seen to be with what went before, and of how physicists have not yet really digested it.

The first chapters take the reader for a race, a bit breathless, through some of the main ideas of classical mechanics, relativity and quantum theory. We are told of how physicists have come to think of the world as a "game of chance in which we are not merely spectators but players" — in that we now have fundamental laws which are indeterministic in character and whose formulation (in the orthodox contemporary view, in so far as it is coherent) seems to require reference to the observer; of the uncertainty principle and the exclusion principle; of the Dirac sea and the antiparticle; of how space-time itself must be subject to quantum fluctuations and be 'foamy' on a small scale. Repeatedly we are told how very bizarre and incomprehensible it all is.

In later chapters the story unfolds of the remarkable ideas advanced by remarkable physicists in trying to comprehend it all — Bohr's idea that we must renounce all attempts to visualize the atomic and subatomic world and must be content with a formalism that allows statistical predictions to be made about macroscopic events in macroscopic equipment; Wigner's concept that human consciousness plays an essential dynamical role in the world, in 'reducing the wave function'; Wheeler's idea that the very existence of the world, at any time, may somehow be caused by the existence of an observer at some time; the 'anthropic principle' of Dicke and Carter, that much in the observed world is fixed by

Other Worlds: Space, Superspace and the Quantum Universe. By Paul Davies. Pp.208. (Dent: 1980.) £7.50.

the fact that it is being observed, because with even minor changes the world could not have produced observing creatures like us.

Above all we learn of the 'many-universes' interpretation of quantum mechanics, of Everett and De Witt, according to which all universes that might exist do exist. If they all exist, then the existence among others of relatively few tailored for our comfort is no mystery, and of course it is only in one of these few that we could find ourselves. Professor Davies is clearly attracted to the many-universes interpretation — and derives from it the title of his book — but correctly makes clear that it is taken seriously only by a handful of physicists. A final chapter dwells on the mystery of time, and especially the absence from our equations of anything corresponding to our impression of the passing of time. One must admire the intellectual intrepidity, and subtlety, of the author in "finding no reason to suppose that the future does not already exist, even though it is not yet determined, and even though the observer will have a hand in structuring it . . .".

All this is vividly done. Perhaps even a little too vividly. Sometimes the confidence, even the complacency, of the assertions can be irritating. "We must acknowledge . . ." and "We have seen that . . ." really mean only that some qualitative considerations might suggest the entertainment of one possibility among others. But often the unqualified statement is modified a little later, or even has been qualified earlier — a distinction perhaps of little moment to one with such an Olympian view of time. In the closing sentences of his book the author allows that our subjective impression might be more reliable as regards time's passing than our equations. And his assertion that most scientists would agree that ". . . past, present, and future are merely linguistic conventions . . ." has been qualified already in the prologue, where it is said of such ideas that "most scientists live a sort of dual life, accepting them in the laboratory, but rejecting them without thought in daily life". Yes indeed.

On the whole the book gives a good impression of what is thought about these

questions by physicists who consider them. But there is an exception, and it is a serious one. It concerns the views of Einstein in general and the Einstein-Podolsky-Rosen argument in particular. As regards Einstein's general reservations about quantum mechanics, Professor Davies propagates the view that they centred on indeterminism: "So unpalatable did this inherent chanciness seem to Albert Einstein that he refused to believe it throughout his life, dismissing the idea with the famous retort 'God does not play dice'". Perhaps the quotability of this famous retort is partly to blame for the fact that Einstein's more considered views are less well known. Compare the summing-up above with the following by Pauli. He is writing to Born in 1954:

. . . Einstein does not regard the concept of 'determinism' to be as fundamental as it is frequently held to be (as he told me emphatically many times) . . . he *disputes* that he uses as a criterion for the admissibility of a theory the question: — Is it rigorously deterministic? — . . . he was *not at all* annoyed with you, but only said you were a person who will not listen".

The Einstein-Podolsky-Rosen (EPR) argument comes up in Chapter 6: "So far we have deliberately been rather cavalier about notions like the real world. . . . In this chapter we shall face up squarely to the fundamental questions raised by the quantum revolution . . .". In particular, we face up squarely to the possibility that "the apparently capricious and random fluctuations of microsystems do not represent an intrinsic indeterminacy in Nature, but are simply manifestations of a hidden level of structure . . .". And then the EPR correlations are presented as the fundamental argument against such a possibility! While for EPR they were an entirely convincing argument for precisely such a possibility! The discussion exhibits the perplexity of one who refuses to contemplate the possibility that quantum mechanical description, of a photon in particular, is incomplete. It was the position of EPR that such perplexity could be relieved just by admitting the hypothesis in question. That Professor Davies misses the point not only of the original work, but of the renewed discussion and the associated experimental programme, is clear from the following: "The truly mind-boggling implications . . . are apparent if we use two parallelly orientated polarizers . . .". On the contrary, the mind is boggled only by

polarizers which are not parallel. The results with parallel polarizers are easily explicable along Einsteinian lines. The results with off-parallel polarizers are not. That is the new point, unknown to EPR, which inspired the experimental programme of recent years to which the author seems to refer.

While I claim that Professor Davies is

wrong on these points, it would certainly be unjust to pillory him on account of them. He makes these mistakes, if they are indeed mistakes, in the company of many other distinguished physicists. He could claim to be reporting a consensus! I enjoyed the book, despite all the reservations, and expect it to be enjoyed by many. The author provides a lively account, accessible

with very little technical background, of what is being said about some momentous and inevitable questions. And if he sometimes leads into error, he does not lead into a minority. □

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Data book on zinc

Roy M. Harrison

Zinc in the Environment. Edited by J. O. Nriagu. Part 1 *Ecological Cycling*, pp.453; Part 2 *Health Effects*, pp.480. (Wiley-Interscience: 1980.) Each volume £27.50, \$59.85.

MOST non-specialists will probably associate the subject of metals in the environment with possible adverse human health effects; indeed in the cases of metals such as lead, cadmium and mercury the main stimulus for research has arisen from concerns over toxic symptoms arising from excessive environmental exposure. Zinc provides a very different case, however. Adverse health effects found in the general population result more often from zinc deficiency, rather than from toxicity due to elevated exposure. Indeed, despite the ubiquitousness of zinc in our environment, numerous well-documented cases of serious zinc deficiency syndrome have arisen amongst groups of people in many different parts of the world. Instances of

zinc intoxication in humans are comparatively rare, arising only from extreme exposure conditions not normally experienced by the general population, and in some instances probably due to the cadmium impurity invariably present in technical-grade zinc.

The essentiality of zinc in mammalian metabolism coupled with its considerable toxicity, especially to aquatic biota, make the study of zinc in the environment an important matter which has been rather neglected in comparison with the intensive research efforts expended upon other environmental metals. This two-volume compilation by Nriagu may help to redress the balance; it is an extremely valuable collection of both factual information and numerical data on environmental zinc, and provides a useful source book.

Part I covers the production and uses of zinc, the environmental concentrations and pathways of the metal and its cycling within, and effects upon, near-shore marine, freshwater and forest ecosystems. Part II deals primarily with human health effects of zinc, but also includes the effects of zinc upon terrestrial and aquatic flora and fauna.

As in other recent books edited by Nriagu, the emphasis is upon detailed, but frequently uncritical reviews of the literature. Contributions are drawn from a large number of specialist authors and are fairly well up to date, with references up to 1978 included. The editor has imposed a fairly high degree of uniformity of style, and the book gives wide coverage of the topic with few omissions or overlaps. In these regards the book is of far greater value to the reader than the now-common review volumes cobbled together from the proceedings of a symposium and purporting to provide complete coverage of a topic.

Zinc in the Environment cannot be recommended as light reading for the individual requiring a brief overview of the subject. It will, however, be regarded as essential by many research workers in the field and will, in my view, represent an invaluable source of information for a good many years to come. □

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En route to a classic of ornithology

Raymond O'Connor

Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic. Vol.II. Edited by Stanley Cramp. Pp.695. (Oxford University Press: 1980.) £30, \$85.

THIS is the second volume of *The Birds of the Western Palearctic*, the ambitious multi-volume work intended as the successor to Witherby's *Handbook of British Birds*, though with a wider geographical brief than the British Isles of the 1939 work.

The success of Vol.I testifies to the overwhelming acceptance of its general approach and format, and Vol.II follows the same pattern. The volume treats those species belonging to the Accipitriformes, Falconiformes, Galliformes and Gruiformes. Each species account begins with a general consideration of field characters followed by detailed discussion



of the plumage characteristics of the recognizable age-classes. Sections on habitat, distribution, population, movements, food, behaviour and breeding follow, and each account is concluded with a listing of plumage and morphology data. The approach is entirely specific except for brief introductions to each family. The editors have rightly persisted with the format of Vol.I with only minor modifi-

cations, though I wish they would quote author initials when citing authors of the same surname. Another minor irritant is the failure to reproduce the explanatory material given in the first volume. As a reference work the *Handbook* should not require one to consult two hefty tomes to locate one's target information within Vol.II.

One general feature of this work is that its treatment of a topic is determined very much by the nature of the material available. For the kestrel, *Falco tinnunculus*, for example, nest site frequencies are tabulated for German and British studies, regional differences are described between northern and southern Britain, seasonal variation in clutch size is cited on the basis of a Swiss study, egg loss rates are quantified for Dutch and English populations, and breeding success to food supply correlations are mentioned for a Norwegian study. With the Western Palearctic as their geographical remit, the authors are entitled to their use of studies from different areas but I find two shortcomings in this approach. First, is the reader to assume that effects cited in a regional study are common to the whole

region? The editors disclaim the validity of such assumption from the outset, correctly cautious but unhelpful to the reader. May we, for example, assume that British kestrels follow the Swiss pattern of a seasonal decline in clutch size or is it the case that the question has not been examined for the British population? Second, the absence of reference, for example to habitat differences in clutch and brood sizes, means what? — that there are no such differences or that there are no studies of this aspect of the species' breeding biology? With so much of the volume's layout standardized I regret the lack of systematic treatment of these finer points.

I also have mixed feelings about the treatment of species behaviour, mostly as to whether or not the text devoted to the subject is in keeping with that afforded more ecological topics. On diet, the authors have avoided the presentation of anecdotal data forced on Witherby, so thankfully the literature will be spared a further succession of notes of the 'Additional food items taken by the Greater Gas-bill' type. With behaviour, however, this danger has not been avoided as successfully. Having said this, I admit fully the usefulness of having the lucid descriptive accounts of what is known of the various species. Whilst some species have extensive coverage, for example seven full pages for the peregrine, *Falco peregrinus*, many run to only one or two columns of text and provide a valuable indication of the gaps in our knowledge.

The line drawings of C.J.F. Coombs illustrating the sections on behaviour are, on the whole, successful in providing schematic illustration of the behaviours of interest, without at the same time losing all of the natural 'jizz' of the birds. He deserves special credit for meeting a difficult task well. The colour plates are beautiful and for the game birds, especially, convey real expression. I was less taken by the plates illustrating perched raptors and was reminded by several of the 'in flight' illustrations of this group of nothing so much as of aircraft recognition charts. I personally would find the Peterson *Field Guide* (Collins, 1954) technique of pointers directed to the critical recognition features of help in using these illustrations, despite the reference rather than 'in field' purpose of this book.

If the work has a significant weakness, as opposed to the more trivial criticisms mentioned above, it is in relating the fieldwork and natural history to a broad theoretical background. Within its taxonomic scope this volume provides a magnificent synthesis of facts but not of knowledge. Examples abound. Female raptors are noted as taking a larger share of territorial defence as the nest cycle progresses from laying through to nestlings, but nowhere is there reference to the parental investment theories of Trivers nor to the study of Barash (*Wilson Bull.* 87, 367–373;

1975) whose paper makes explicit the relevance of these theories to the nest defence pattern. Similarly, the parent–young bonds of the sparrowhawk, *Accipiter nisus*, are described as being typical of *Accipiter* in character and that of kestrels as 'largely typical of falcons' but the reader is left to his own initiative to return to the family introductions to seek a description of these typical patterns. Even where general patterns are recognized as existing and deserving of theoretical



explanation, as with the reversed sexual size dimorphism of the accipiters and falcons, the authors duck the question, giving references without even a hint of the nature of the theories to be found therein. This refusal to acknowledge that there is a 'Why' to the 'Whats' of bird-watching is, to my mind, the great weakness of this

book. It undoubtedly reflects the composition of the editorial board for the *Handbook* and their individual interests, as well as the composition of the 'serious bird-watcher' market at which it is aimed. However, if ornithology has (as I believe to be the case) more to offer to the ecological and evolutionary sciences than has the study of any other single taxonomic group (with the possible exception of entomology) the replacement for Witherby's *Handbook* ought to display at least a nodding acquaintanceship with these sciences.

How easy it is to criticize! The volume lives up to the standards set by Vol. I and, in turn, by the Witherby classic. It brings so much information together that I could not be without a personal copy of this storehouse of avian natural history. Put alongside a copy of Welty's *Life of Birds* (W.B. Saunders, 1975) it will provide the serious bird-watcher with most of what he should know about the groups covered. My real regret is that at the present rate of writing (with three years between the publication of Vol. I and II) it will be 1995 before an authoritative compendium on the passerines is available. On their form to date Cramp and his colleagues will then have completed a classic of ornithological compilation. □

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A plea for 2,4,5-T

Alastair Hay

The Science of 2,4,5-T and Associated Phenoxy Herbicides. By Rodney W. Bovey and Alvin L. Young. Pp.462. (Wiley: 1980.) £21.40, \$46.90.

"FEW agricultural chemicals have a longer safety record in the field than the phenoxy herbicides, including 2,4,5-T". This conclusion, stated baldly by the authors in the opening pages, sets the tone for this, the latest book on the subject.

As the authors note in their first chapter, the use of 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) has been a controversial matter in many countries for the past ten years. That controversy has centred, in the main, on the presence in 2,4,5-T of an extremely toxic contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin). The dioxin contaminant, produced as an unwanted by-product in the manufacture of 2,4,5-trichlorophenol — from which 2,4,5-T is made — is a known teratogen and carcinogen in animals. However, dioxin is only present in most 2,4,5-T preparations in concentrations less than 0.1 ppm and in Britain, according to a recent government statement, in amounts less than 0.01 ppm.

Is all the fuss about 2,4,5-T justified? Bovey and Young think not. It is their view that much of the criticism of the herbicide is misinformed and based on biased, selective citation of reports. The authors consider many of these reports in some depth, and present evidence to show that allegations against 2,4,5-T are ill-founded. For example, two incidents linked to 2,4,5-T — an alleged increase in human illnesses in Globe, Arizona, and the deaths of reindeer in Lapland, both of which occurred in 1970 — were later shown to have no connection with the herbicide. Local doctors practising in the Globe area said that close investigation revealed no significant increase in illnesses linked to herbicide spraying. And the reindeer deaths in Sweden were eventually attributed to starvation rather than exposure to 2,4,5-T.

To support their argument the authors have provided a detailed review of the chemistry, toxicology and ecological effects of the compounds in question. The value of their work lies in its comprehensive coverage of the literature describing the properties of 2,4,5-T and the related herbicide 2,4-D (2,4-dichlorophenoxy acetic acid) in microorganisms, higher plants and animals, including human beings. Methods of detecting the herbicide in biological specimens are considered, as are the problems associated with its application.

Inevitably, authors of a book such as this

must at some time make a decision when to stop reviewing the literature. Here, with coverage up to 1978, they have therefore missed some of the more important epidemiological studies on dioxin exposure in industry and silviculture.

Recently published studies of people exposed to dioxin in industry offer apparently conflicting evidence. The first investigation, of workers exposed to the chemical at the Monsanto company in West Virginia in 1949, clears dioxin — for the moment — of causing any increase in the mortality rate in the men; deaths in this group were in fact lower than would be expected. The second study, of workers exposed to dioxin in an accident in West Germany, suggests that the chemical could be the cause of an increase in gastrointestinal cancer. A third survey, of workers employed in the forestry industry in Sweden, also claims that exposure to dioxin-contaminated 2,4,5-T could have increased the incidence of deaths from cancer. The authors of this study claim that silviculture workers exposed to 2,4,5-T have a six-fold higher incidence of soft tissue sarcomas (see *Nature* 283, 613;

1980).

In their concluding chapters Bovey and Young detail the processes involved in registering new chemicals and list many suitable alternatives to phenoxy herbicides — although, as stated earlier, their view is that the phenoxy herbicides are not a great hazard. The economic consequences of dispensing with them is assessed as follows: an increase in farm production costs of \$290 million, and the requirement of an additional 20 million hours of family labour to clear the foliage the herbicides keep down so effectively. However, it is not clear whether these figures are based on a situation where alternative herbicides are employed or one where no herbicides are used at all.

On phenoxy herbicide use for military purposes, the authors are quite explicit. Their use "in an armed conflict [in Vietnam] has jeopardised their continued use in world agriculture". The authors are not convinced that the use of 2,4,5-T in Vietnam — recognized to have had a dioxin content of 0.05–47 ppm — has caused birth defects among the general population, as has been claimed by Vietnamese scientists

and doctors. However, Bovey and Young note that many scientists still hold different views on this subject and that more evidence is required before the issue can be resolved. The authors also claim that the incineration of the unused herbicide stocks from Vietnam means that "it is no longer an issue". Using herbicide destined for Vietnam may not be an issue — but the legacy of its use in that country is still very much in the public eye, following claims by former US veterans that the herbicide has damaged their health.

It remains to be seen whether reviews currently being conducted by government bodies in the UK and USA will come to the conclusion that the use of present formulations of 2,4,5-T also poses a health risk. Whatever the verdict, the authors make the plea that the suspension of 2,4,5-T and other products based on trichlorophenol "should not jeopardise other phenoxy herbicides". By all accounts the others are not in jeopardy. □

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Odours and vertebrate ecology

Vincent B. Wigglesworth

The Ecology of Vertebrate Olfaction. By D. Michael Stoddart. Pp. 234. (Chapman and Hall: 1980.) £15, \$29.95.

THE purpose of this book was to put together an inclusive account of all the ways in which the lives of other vertebrates are guided by the richly scented world that is beyond our ken. Comparative anatomy of the olfactory system suggests that we differ from our more osmotic relatives in quantity rather than in quality; in the restricted area of our olfactory epithelium in comparison with the capacious chambers of other animals. As far as sensitivity is concerned, it sounds quite impressive to point out that the human female at the height of her sexual cycle can perceive the odour of synthetic musk at a concentration of 1 in 1,000 million; but this achievement fades beside the claim that the eel can detect β -phenylethyl alcohol at concentrations as low as $3 \times 10^{-18}M$, representing the dilution of 1ml of the alcohol in a volume of water equal to 58 times that of Lake Constance (Bodensee, the 60km lake on the Swiss–German border) — which will provide the eel with about three molecules in its olfactory sac at one time.

A survey of the chemistry of vertebrate scents reads like a catalogue of organic chemicals, but on closer analysis it seems

that virtually all are refined waste products of metabolism and few or none are synthesized *de novo*. Moreover, although the glands from which they are liberated can be concentrated in many different parts of the body, they seem always to be modified, often scarcely modified, sebaceous glands, the secretory products of which are influenced by diet.

The book provides a detailed account of the multifarious roles of olfaction — the detection of food by herbivores and carnivores; the attraction of a mate and the advertisement of sexual state; the induction of oestrus and ovulation; the conduct of courtship and mating; the discrimination of families, populations and races (thus aiding sexual isolation between species); the maintenance of social hierarchy and territoriality; the detection of predators; the transmission of warning signals or active defence; and homing and navigation.

Olfaction is greatly involved in reproduction — in sexual attraction or the excitation of females by the rutting male, which may induce simultaneous oestrus in a wide group of females. In that most human of animals, the tortoise, the female in the early stages of courtship rubs her forearms against her chin glands and then proffers her perfumed arms to passing males. Even pregnancy may be upset by olfaction: in female mice the presence of a strange male or just his urine may prevent implantation of existing blastocysts in the female and may even terminate pregnancy more than half-way through gestation. Also, after birth, odour plays an essential role in forging the bond between mother and young.

Olfaction can be important in the isolation of species: among mice the odour preference by the female seems to accord with sound genetic policy in that she prefers an unrelated male of the same stock, avoiding males of remote strains and rejecting siblings.

The author naturally devotes much attention to the marking of territory — a world of sense and a complex language which it is difficult for us to understand. It would seem, in general, that marking does not serve to keep intruders out but acts rather as a bond within the colony which boosts self-confidence and thus the ability to get the better of unwelcome intruders.

In a final chapter the author outlines some of the ways in which an understanding of the role of olfaction in the ecology of vertebrates may be utilized in improving husbandry or combating vertebrate pests. He does not claim too much. But in his eyes the grass looks greener on the other side of the fence; he gives a brief account of the use of pheromones in insect control which might fairly be described as optimistic.

This book is packed with information, well organized and fully documented with references. The author favours theories based on observational or experimental science. He is sceptical of conclusions based on anecdotes and pays only minimal attention to Freudian fantasies. He is therefore a good guide for the serious student of ecology. □

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